THE EFFECT OF EMAPII ON DENDRITIC CELLS

By

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EFFECT OF EMAPII ON DENDRITIC CELLS

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List Of Abbreviations

APC	Antigen Presenting Cells
BGS	Bovine growth serum
BMDC	Bone marrow derived dendritic cells
CD40L	CD 40 ligand
DC	Dendritic Cells
EMAPII	Endothelial Monocyte Activating Polypeptide II
FITC	Fluorescein Isothiocyanate
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
IFN α	Interferon Alpha
IL3	Interleukin 3
IL5	Interleukin 5
IL7	Interleukin 7
IL10	Interleukin 10
IL12	Interleukin 12
LC	Langerhans Cells
LPS	Lipopolysaccharide
MethA	Methylcholantharene Induced
MHC I	Major Histocompatibility Complex I
MHC II	Major Histocompatibility Complex II
MIP 1aplha	Macrophage Inhibitory Protein 1 alpha
PBS	Phosphate buffered saline
STAT3	Signal transducers and activators of transcription 3
TIDC	Tumor Infiltrating dendritic cells
TLR	Toll like receptor
TNF	Tumor Necrosis Factor

CHAPTER I

REVIEW OF LITERATURE

Immune System at a glance

Our body is invaded by myriads of organisms on a daily basis. In order to combat these invaders, an extremely complex and sophisticated network of cells and mechanisms collectively termed as the immune system is in place. The immune system is divided into two main branches: innate immunity and acquired immunity. Innate immunity entails the defense mechanisms that an individual is born with and acquired immunity is the immunity that an individual obtains during the course of his/ her development. When the body encounters a pathogen, the first line of defense is mounted by the innate immune system to eliminate the invading organism/source. When the innate immune system fails, the acquired immune system is triggered to purge the invasion. Some of the major cells that are a part of the immune system include T and B lymphocytes, neutrophils, basophils , macrophages, monocytes, eosinophils and dendritic cells.

Hematopoiesis involves the development of immune system cells, platelets and erythrocytes from primitive stem cells and this process usually occurs in the bone marrow (1). Almost all of the immune system cells arise from a common hematopoietic stem cell or HSC. This stem cell divides to generate a progenitor cell from which different cell types originate. The cells of the immune system are derived mainly from one of the two

main lineages: the lymphoid lineage and the myeloid lineage. The third lineage is the erythroid lineage which is a source of erythrocytes and platelets. The lymphoid lineage serves as a source of lymphocytes and natural killer cells which are the main players of adaptive/acquired immunity whereas the myeloid lineage yield inflammatory cells and cells of the innate immunity branch.

Progenitor and precursor cell differentiation is governed by the presence or absence of a variety of cytokines released by bone marrow stromal cells. Hematopoietins are the major class of cytokines that drive the differentiation of blood cells in the bone marrow. Some of the other cytokines that play a significant role in directing the differentiation include colony stimulating factors and interleukins. The differentiation of some of the cells is completed in the bone marrow. However some cells have to pass through additional stages to complete maturation. They might leave the bone marrow as immature cells and complete their process of maturation at distant sites or locations.

Dendritic Cells

Dendritic cells, often called as ontogenic orphans, are professional antigen presenting cells (APC) that are rare but ubiquitous. Identified by Steinman and colleagues in 1972,these cells are the sentinel cells of the immune system(2). Professional antigen presenting cells capture antigens, process them and present them to the lymphocytes which in turn activate the lymphocytes. They arise from hematopoietic stem cells and exhibit characteristic dendritic morphology and strong antigen-presenting capacity (1). They do not exhibit lineage-specific markers like CD3,CD19,CD16 and CD14 which are expressed respectively by T cells, B cells, NK cells and monocytes (1). DC can follow either one of the two developmental lineages; lymphoid or myeloid. They

differ in their function and surface marker expression. Lymphoid DC express the marker CD8α which is absent in myeloid DC. Lymphoid DC are mainly involved in immune tolerance and myeloid DC capture antigens in the periphery, migrate to secondary lymphoid organs and activate specific T cells (3-4).

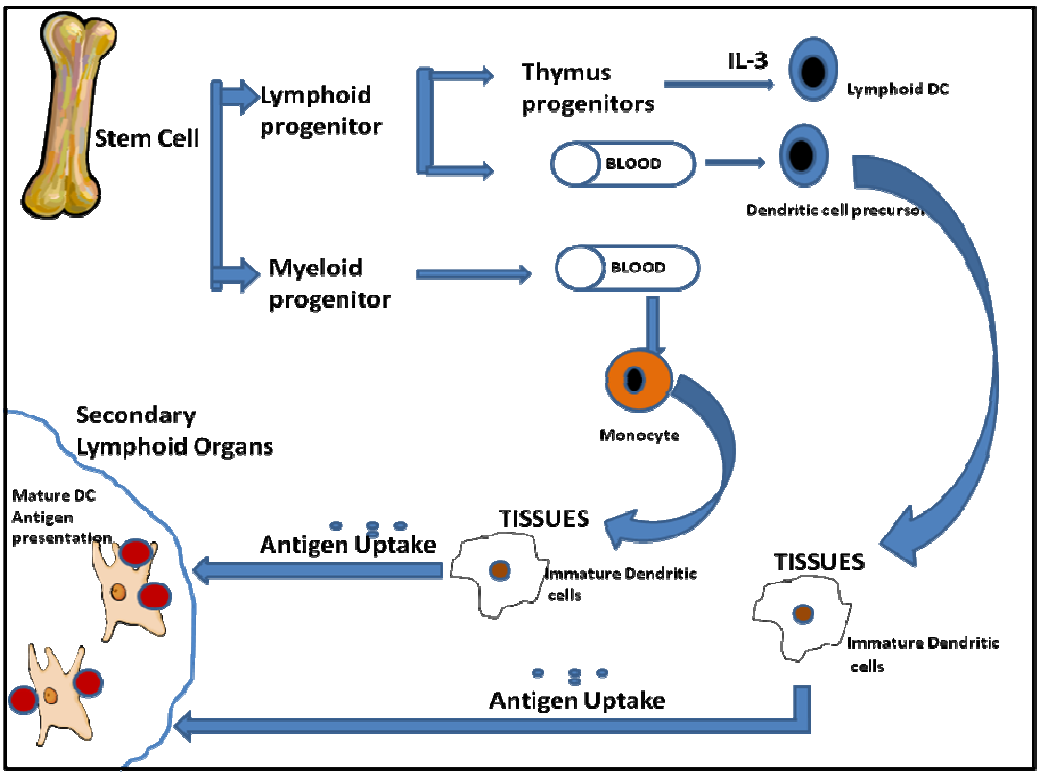
Dendritic cells are extremely versatile cells of the innate immune system with distinct functions and exhibit several distinct forms. They are distributed in a variety of tissues and organs including non lymphoid tissues like skin, mucosa, organs like the heart and the kidneys, as well as peripheral blood and lymph. The prime location of dendritic cells is the T cell dependent areas of lymphoid tissue (5). Dendritic cells residing in the epidermis are called Langerhans cells while the dendritic cells populating the T cell rich region of secondary lymphoid organs are called interdigitating dendritic cells (6). The other locations include the thymic medulla and B cell follicles of secondary lymphoid organs. DC residing in the dermis are known as dermal dendritic cells or interstitial dendritic cells. There is also a very distinct group of cells that are characterized as dendritic cells but cannot present antigens efficiently to naïve T cells (7). These are called the plasmacytoid dendritic cells. They play an important role in mounting adaptive responses to viral infection. They have a larger repertoire of IFNA genes compared to other dendritic cells and secrete enormous amounts of IFN-α in response to a variety of viral and non viral stimuli (8). Plasmacytoid DC lack cd11c or express very low cd11c, express TLR9 and TLR7 and are involved in the development of regulatory T cells and tolerance (8-9).

Developmental Cycle of Dendritic Cells

Dendritic cells also originate from the bone marrow during hematopoiesis (2). There are distinct hematopoietic lineages of DC. This includes lymphoid related DC, monocyte derived DC and myeloid DC. A common lymphoid progenitor cell is the ancestor of lymphoid related DC. These cells are inept at differentiating into monocytes. Monocyte-derived DCs are generated by monocytes. This transition is mainly caused by GM-CSF and IL-4 (1).The phenotype of these cells is transient. Myeloid DC arise from clonogenic precursors distinct from the precursors of myeloid cells. These cells develop independently and distinctly from monocytes (1). Dendritic cell precursors leave the bone marrow to populate different organs and tissues.

Dendritic cells usually reside as immature cells at different tissues and interstitial spaces. They mature when exposed to a variety of stimuli. Mature and immature dendritic cells exhibit characteristic differences in terms of cell surface expression and functions. Immature dendritic cells have high amounts of intracellular MHCII proteins but they are retained within the cell with very little surface expression (10). They are extremely efficient in taking up antigens and cellular debris via receptor mediated endocytosis, phagocytosis or micropinocytosis. In the immature form DC express CD1a and have very low capacity for T cell stimulation. They lack adhesive and co stimulatory molecules like CD40/80/86 required for activation of T cells. They also have low levels of CD25,DEC-205(10-11).However, they express receptors for inflammatory cytokines like CCR1. Immature DC constantly scans their surrounding milieu for invading organisms or foreign particles.

A maturation signal is usually received from local infection. Upon maturation, dendritic cells change their morphology. They exhibit dendrites or have a veiled appearance. They upregulate receptors that are required for migration, such as the CCR7 receptor. MHC I and II are expressed at high levels on the surface. They lose their phagocytic ability and acquire co stimulatory molecules like CD80/86/40 that are required for activating T cells (10). These cells then leave the tissue/organ where they were residing in and migrate to secondary lymphoid organs where they present the captured antigens to the resident T cells. Maturation of dendritic cells is a constant process which is not dictated by the presence of infection. Dendritic cells can mature in the absence of infection and these cells do not mediate effector differentiation of naïve T cells.



Immune regulation of dendritic cells is mostly via programmed cell death or apoptosis which is an extremely regulated and controlled event. DC undergo apoptosis if they fail to receive signals from the T cells (3). Apoptosis is a part of the developmental cycle of DC. DC can undergo apoptosis during three different phases of their development. These include the initial progenitor stages where the progenitors that failed to undergo further development underwent apoptosis, intermediate stages during precursor selection and finally at the mature level after activating T cells or in the absence of appropriate signals (10).

Functions of Dendritic Cells

DC are the guard cells of the immune system. They constantly scrutinize their environment for antigens. Even under homeostatic conditions, there is a small rate of DC turn over. Even in the absence of inflammation or antigen stimulation, DC migrate from the peripheral tissues to secondary lymphoid organs (12-13).

Antigen acquisition

As antigen presenting cells, DC are required to capture antigens, process them and present the processed peptides to T cells (14). Dendritic cells capture antigens by a variety of methods. They can internalize antigens by means of receptor mediated endocytosis or phagocytosis (15).When receptor mediated uptake fails, DC can still internalize antigens via pinocytosis which involves micropinocytosis and macropinocytosis (16). Almost all cells are capable of micropinocytosis however macropinocytosis is a special function possessed by only a few cells. Dendritic cells can efficiently take up macrosolutes via macropinocytosis and concentrate them in MHC loaded compartments (16).

Antigen Processing

MHC molecules are a group of proteins that are encoded in the major histocompatibility complex .These proteins play important roles in governing the immune responses. These proteins bind to peptide fragments of proteins internalized by the cell and display the degraded peptide on the surface for T cell recognition and activation. Naive T cells can be activated only if the peptides are presented in context with an MHC molecule (17). There are two major classes of MHC molecules; MHC Class I and MHC Class II. They differ both structurally and also with respect to the peptides they bind to. CD4T cells can recognize antigens when they are in context with MHCII whereas CD8T cells can recognize only MHC class I associated peptides.

Within a cell there are two main areas that need to be surveyed for infection. This includes the cytosol which is the potential site for viral replication and intracellular bacteria and endosomal/lysosomal compartments that harbor the antigens derived from internalized extracellular pathogens and bacteria.MHC class I molecules bind to the peptides derived from the cytosolic compartment whereas MHC class II is associated with sampling the endosomal/lysosomal compartment (11). MHC class I molecules are expressed by almost all the body cells with an exception of erythrocytes. However, expression of MHC class II molecules is restricted to only specialized cells like antigen presenting cells and phagocytic cells. Dendritic cells are capable of processing antigens and presenting them in context of MHCI and MHC II indicating their ability to prime both CD4+ and CD8+ T cells (18).

The peptides derived from endogenous antigens degraded by the proteosome complex in the cytosol are transported to the endoplasmic reticulum where they are

loaded onto MHC class I proteins. The assembly then proceeds to the cell surface for display. The MHC class II processing takes place initially in the endoplasmic reticulum where the MHCII molecules assemble. They are then chaperoned to the endosomal/lysosomal pathway wherein they bind the peptides derived from exogenous antigens. The entire complex is transported to the surface for expression (11, 19).

Dendritic cells are highly efficient in antigen processing and presentation. They are capable of forming MHC-peptide complexes from internalized cells. In the absence of a signal the MHC II expression on immature DC is kept minimal. Upon antigen stimulation, the peptide loaded MHC II that reach the surface remains on the surface with the shutting down of further MHC II production (11).These peptides are then presented to T cells.

Dendritic cells can effectively process exogenous antigens and load them in MHC class II complex for presentation.

Antigen Presentation

Dendritic cells are called professional antigen presenting cells since they are the only cells that can activate naïve T cells. The processed peptides with the MHC complex are displayed for T cell receptor binding. The T cell receptor must bind to the peptide-MHC complex with high affinity so that the T cell receptor signal is prolonged enough to initiate further events downstream. This requires involvement of co receptors and co stimulatory molecules present on both T cells and dendritic cells. Some of the co stimulatory molecules include CD80 and CD86 on the dendritic cells.

Mature dendritic cells are also capable of cross presentation wherein the peptides derived from the endosomal compartments are loaded in to MHCI molecules as well.

Thus dendritic cells can activate both CD8 and CD4 T cells against peptides derived from the same antigen/organism.

Dendritic cells also play a role in the induction of tolerance. Dendritic cells can process antigens derived from apoptotic cells and present them in context with MHC class molecules. This would either stimulate or tolerize CD8 positive cytotoxic lymphocytes (20-21). Tolerance can be induced only via apoptosis since necrosis can bring about a stimulatory response (20). There is a reasonable argument that migratory immature dendritic cells respond to apoptotic cell death in the peripheries. After capturing the apoptotic bodies, these immature DC migrate to the lymph nodes wherein they present the captured antigens to DC housed in the lymph nodes (22). The resident DC processes the short-lived migratory DC and induces tolerance. These DC are sometimes referred to as regulatory/tolerogenic DC and exhibit high MHC-peptide complexes and low CD86. They cannot drive T cells through a stimulatory pathway. DC are required for negative selection and not for positive selection (22).It is proposed that peripheral tolerance mediated by migratory DC complements central tolerance. Peripheral tolerance seems to be essential to prevent autoimmune responses against harmless environmental and tissue antigens that do not gain access to the negative selection process in the thymus. When low doses of soluble antigens are encountered by DC in the steady state they induce peripheral tolerance to these antigens (22). T cells that interact with tolerogenic DC get deleted after few rounds of division. Tolerogenic DC exhibit low levels of co stimulatory molecules, low allostimulatory activity , low CCR7 expression and release IL-10 which is an immune suppressive cytokine (9).

Migration

Even though migration cannot be considered as a function it is a very important aspect of DC development. Dendritic cells migrate from the tissues where they reside in or peripheral blood to various lymphoid organs (23). One of the major routes of DC migration is from the peripheral tissues to the draining lymph nodes (24). Migration of dendritic cells is a very critical process and is tightly regulated. DC migration is critical owing to the fact that T cells are usually found in the T cell rich regions of secondary lymphoid organs. DC have to reach T cell regions in order to deliver the signals and activate the T cells to mount an immune response.

Chemokine and Cytokine Secretion.

Cytokines are low-molecular-weight proteins released by immune system cells and other cells of the body. Cytokines play important roles in the development and effector functions of immune system cells. Unlike hormones, cytokines exert their effects locally over short distances. They are intercellular messengers that relay information that are vital for the functioning of the immune system and could bring about proliferation, activation, differentiation, immunoglobulin class switching or block all of the above in target cells (25). Chemokines are cytokines that are chemotactic. They are small proteins and are characterized by the presence of four cysteine residues in conserved regions.

Dendritic cells are capable of releasing cytokines and chemokines. The array of cytokines secreted by dendritic cells depends upon the stage of the cell. Apart from secreting cytokines, DC also express cytokine receptors which would enable them to respond to the cytokines that act in an autocrine or paracrine fashion. When stimulated with phytohemagglutinin, DC release a wide array of cytokines of which IL-9 and Rantes

are important owing to the fact that they are not released by monocytes (26). Unstimulated DC also exhibit cytokine transcripts although it is not as pronounced as it is in an activated DC. DC are capable of producing multiple cytokines that have multiple functions (27).Of importance is IL-12 which plays a significant role in the development of Th1 cells. DC can produce IL-12 and thus influence the switch between Th1 and Th2 cells (28).

Dendritic Cells and Tumors

Most tumors are poorly immunogenic and thus fail to elicit an immune response. As discussed earlier, DC have strong antigen presenting capacity and are important for activating T lymphocytes. DC are capable of stimulating tumor specific T cells. It has been reported that Langerhans cells can effectively present tumor associated antigens and stimulate CD4+ T cells (29-30). Bone-marrow derived DC can induce both cytolytic and proliferative anti tumor responses from naïve splenocytes. Naïve T cells incubated with tumor lysate-pulsed DC gave a proliferative response (31). These cells could also effectively lyse the tumor cells (31). Apart from stimulating tumor specific T lymphocytes, DC can also be effective for tumor immunization. Immunization of mice with tumor lysates-pulsed DC protected the animal from subsequent tumor challenge (31-32). Also, tumor lysates-pulsed DC could reduce the establishment of distant metastatic foci by 90% (31).

Owing to the fact that dendritic cells can initiate anti-tumor responses, it is not surprising to find that tumor cells elaborate mechanisms to alter DC functions in order to benefit their survival. This alteration could be manifested as a block in the DC infiltration, complete abrogation of phagocytosis, inhibition of DC maturation and /or

obstruction of DC migration into the lymph nodes (33). Gabrilovich et.al showed that tumor cells altered DC differentiation from progenitor cells to suit their survival (34). Bone marrow derived dendritic cells (BMDC) in the presence of viable or irradiated tumor cells exhibited an increase in the cell surface molecule expression (35). Markers like CD80, B7-DC, which would aid in tumor survival, were expressed at higher levels. However, this expression was not as high as compared to that induced by LPS and Poly-IC. Tumor cells interfered with the ability of BMDC to respond to TLR stimuli. Tumor cells partially impaired the up regulation of co stimulatory molecules by LPS and Poly-IC. It is interesting to note that the expression of CD80 and B7-DC were not affected by tumor cells. When incubated with tumor cells these cells failed to produce cytokines even in the presence of TLR agonists like LPS and Poly-IC. Even though irradiated /viable tumor cells could enable co stimulatory expression on BMDC,BMDC failed to produce cytokines necessary for the complete activation of T cells (35). Curiel et.al showed that DC from ovarian cancer tissue expressed B7-H1, a negative regulator of cytotoxic T cells, constitutively thereby affecting their ability to activate T cells (36).

Various tumor tissues such as breast, stomach, lung, bladder are infiltrated with dendritc cells (33). Bell et.al. demonstrated that tumor infiltrating dendritic cells [TIDC] have an immature phenotype compared to the DC in the surrounding tissue and mature CD83+ DC were restricted to the peritumoral regions (37). It was speculated that the increased number of TIDC are due to the chemotactic factors released by the tumor cells. Gabrilovich et.al reported that DC isolated from tumor tissue exhibited diminished capacity to stimulate allogenic T cells and it was speculated that the low MHC class II expression on these cells could be responsible for the impaired APC function. However,

the suppressed phenotype of TIDC could be reverted with the administration of CpG nucleotides and anti IL-10 receptor antibody in vivo and in vitro or by overnight ex vivo culture (38-39) . Similar findings corresponding to TIDC were obtained for circulating DC from tumor patients (40-42). To sum up, TIDC and circulating DC from cancer patients or tumor bearing animals have compromised functions. Having outlined the role played by DC in a tumor milieu, the next section focuses on the main candidate of this research topic namely Endothelial Monocyte Activating Polypeptide II (EMAPII).

EMAPII

EMAPII is a product released during apoptosis and since it is also a product of tumor cells, it may alter tumor directed immune responses. The section outlines the discovery of this protein, followed by its sequencing and in-depth study of the myriads of functions performed by this protein. The discussion then focuses on the therapeutic potentials of EMAPII.

In order to escape immunosurveillance, tumors elaborate a variety of procoagulants which could aid in fibrin deposition and coagulation (43). Even though, this could abet the tumor to mask itself from the host defenses, extensive intravascular coagulation could obstruct the blood flow to the tumor. This would divest the tumor from obtaining essential nutrients. Various cytokines and factors can bring about thrombus formation and intravascular coagulation. Tumor necrosis factor can bring about procoagulant activity in human vascular endothelium (44).

Tumor necrosis factor (TNF), a cytokine primarily released by macrophages, is a key player in apoptosis, inflammation, cell survival and immunity. Induced by endotoxins, this multifunctional cytokine causes necrosis of Meth A sarcomas and other

tumors (45-46).With the support from additional data, the European Agency for the Evaluation of Medicinal Products (EMEA) approved the clinical use of this cytokine to treat soft tissue sarcoma via limb perfusion approach (47). TNF is currently administered in tumor therapy for soft tissue sarcomas, metastatic melanomas and a variety of other tumors (46).

When TNF was infused into methylcholantherene A induced fibrosarcomas, it caused extensive coagulation in the tumor vasculature and obstructed the blood flow to the tumor bed (48). It was hypothesized that tumor cells elaborate factors that prime the response of endothelial cells to TNF. It was observed that methA fibrosarcoma derived tumor supernatant could induce tissue factor in endothelium in response to sub maximal concentrations of TNF(48).

The focus then shifted to isolating tumor derived factors that enhanced tissue factor expression in endothelial cells. This led to the isolation of a 44KD polypeptide from methA derived fibrosarcoma tumor supernatant. This polypeptide was purified and characterized for its effect on endothelial cells in the presence of TNF. It augmented the tissue factor induction on endothelial cells in response to varying concentrations of TNF(49). This polypeptide was named Endothelial and Monocyte Activating Polypeptide I. Another polypeptide was also isolated from methA fibrosarcoma tumor supernatant based on its ability to alter endothelial and monocyte functions. This 22KD polypeptide was called Endothelial monocyte activating polypeptide II (EMAPII)(50). Its unique amino terminal sequence was used to raise antibodies in rabbits and this sequence showed homology to human vonWillebrand antigen II. Purified EMPAII induced procoagulant activity in endothelial cells in a dose dependent manner. The level of tissue

factor mRNA transcripts increased with increasing concentrations of EMAPII suggesting a biosynthetic role. EMAPII was also sensitive to reduction and trypsin treatment. It could also induce procoagulant activity in monocytes which occurred in a time dependent manner and was inhibited in the presence of trypsin. Injection of EMAPII into mouse footpads resulted in an acute inflammatory response characterized by edema and infiltration of mononuclear cells (50).

Structure of EMAPII

EMAPII cDNA was cloned using a portion of the unique NH2 terminal sequence to generate degenerate primers (51-52).Human EMAPII cDNA shared 86% identity with murine EMAPII cDNA with the human sequence containing two additional amino acids. It was observed that the mature EMAPII NH2-terminal sequence was encoded by an internal sequence which led to the hypothesis that EMAPII is generated from a preproprotein; a larger polypeptide. However, hydropathy analysis did not reveal any secreted hydrophobic signal peptide for this protein. This was similar to interleukin 1β that also lacked a classic signal peptide but was released as pre-IL1β, which was later proteolytically cleaved to release mature IL1β. An Asp residue in the P-1 position was necessary for this cleavage and EMAPII protein also had Asp residue in the P-1 position. It was therefore speculated that a cysteine protease similar to the one acting ILβ conversion is responsible for releasing mature EMAPII from its pro-form. A tetra peptide-based inhibitor that mimicked the cleavage site of pro-EMAPII blocked the release of mature EMAPII. Using tetrapeptide inhibitors and recombinant caspases, Behrendorf et.al showed that caspase-7 is the EMAPII cleaving protease (53). However, findings by Murray et.al contradicts the single critical aspartate residue concept suggested

by Kao et.al and disproves that caspase-7 is the primary protease that cleaves pro-EMAPII. In vitro cleavage analysis of radio labeled 34KD EMAPII revealed several intermediate species suggesting that the preprotein undergoes sequential cleavage through the action of multiple enzymes. One possible explanation to this discrepancy in findings could be owing to the fact that Behrendorf et.al did not use purified pro-EMAPII for the assays and also they failed to employ the two heterodimers of caspase-3 and caspase-7(54).

A 5'RACE was performed to assess the transcription sites of EMAPII. EMAPII transcription initiates at three different positions; 15 nucleotides from each other(55). Precursor EMAPII protein is a homolog of p43 component of the multisynthetase complex(56). It is also referred to as AIMP1.Pro EMAPII is related to the C-terminal domain of hamster p43 moiety. When multisynthetase complex was treated with caspase-7 ,it led to the release of EMAPII and digestion of p43.It was also shown that the association of p43 in the complex facilitates the cleavage of this protein to EMAPII since purified p43 could not be completely cleaved to release EMAPII (57).AIMP1 has been shown to exhibit cytokine activities as well. AIMP1 induces the maturation of dendritic cells which in turn swings the immune response to a Th1 response (58).

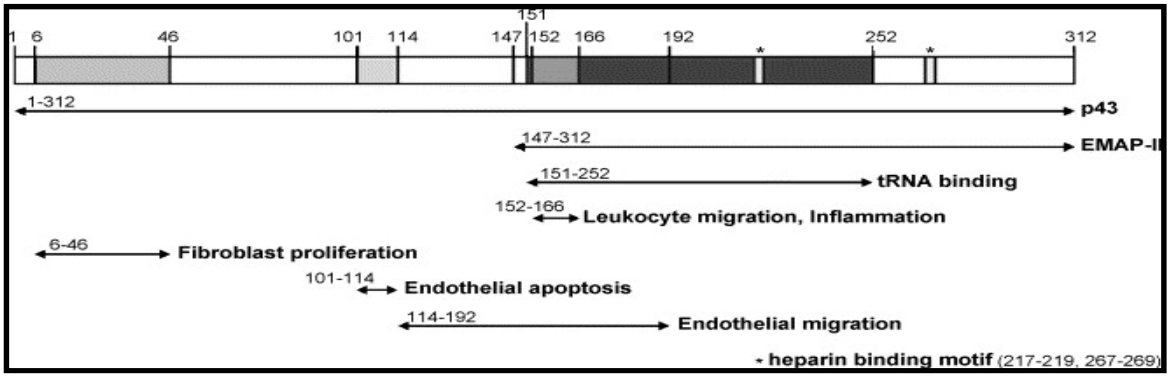


Figure 2: Functional domains of EMAPII and pro-EMAPII

The release of EMAPII is primarily associated with apoptosis and mature EMAPII is secreted mainly during late apoptosis. However, Pro-EMAPII is released constitutively even in the absence of apoptosis. Pro-EMAPII or p43 also possess cytokine activity in terms of TNF-α and IL-8 release. Ko et.al. speculates that cleavage of p43 to release EMAPII is not to activate cytokine activity but to break protein synthesis which would accelerate cell death (59). However, it has been shown that apoptosis is not a prerequisite for the release of EMAPII (60). This paves the way for a reasonable speculation that EMAPII is not just a by-product of p43 cleavage. It has significant biological functions to perform.

Expression

EMAPII mRNA is constitutively expressed in a variety of cell lines and primary cells of human, murine, bovine and simian origin. Primary cells like bovine aortic endothelial cells and mouse embryonic fibroblasts released mature EMAPII when they were subjected to apoptosis (61). This was in accordance with the data obtained using transformed cells that released EMAPII when they were undergoing apoptosis. The release of EMAPII is triggered when the cells are undergoing stress, exposed to hypoxia, in the presence of chemotherapeutic agents or during apoptosis. However, activation of apoptosis is not a prerequisite for this release. Some colorectal cell lines showed cell surface expression of EMAPII. Both soluble and cell surface associated EMAPII are equally active in most of the assays. The 34-KDa form seems to be the prevalent form associated with the surface (60).

EMAPII is expressed at high levels during embryogenesis(61-62). In mouse embryo, high expression of EMAPII mRNA was observed in the midbrain, the neural tube, areas of ossification, interdigital zones and the midgut. The areas in the embryo with high EMAPII expression were found to be undergoing apoptosis at a greater scale indicating that EMAPII co localizes with areas of high apoptosis. In an adult mouse, neurons of the brain, thymocytes of the thymus and germ cells of the testis showed increased expression of EMAPII mRNA. A high rate of apoptosis was observed in the thymus and testis but not in the brain. There is a strong correlation between EMAPII expression and apoptosis in embryonic tissues, testis and thymus. However, this relationship did not hold true for adult mouse brain tissue(61).Immunohistochemical analysis of mouse lung demonstrated that EMAPII is highly expressed in developing lung tissue, however its expression dwindles throughout adult life(63).

EMAPII expression in normal human tissues is high in tissues with high turnover and high protein synthesis (Figure 3). No cellular EMAPII signaling was observed in normal human tissues. This could be owing to the fact that EMAPII being a protein with proinflammatory properties, its release is tightly regulated(54).

System	Cells and Organs	EMAPII expression (protein)
Cardiovascular System	Smooth Muscle Cells	Weak
	Endothelial cells in the heart	Weak
	Blood Vessels	Negative
Gastrointestinal Tract	Salivary glands: luminal surface acini	 Weak negative
	Esophagus	Weak
	Peptic cells of the stomach	Strong
	Parietal cells	Negative
	Hepatocytes in the liver	Strong
	Islets of langerhans cells	Moderate
	Large and Small intestine	Weak
Respiratory Tract	Lung parenchyma	Negative
	Mucinous glands	Negative
	Alveolar macrophages	Weak
	Pneumocytes	Negative
Musculoskeletal System	Skeletal Muscle Cartilage Bone	Negative
Renal System	Kidney proximal tubules glomerular endothelium	 Moderate Negative
Reproductive Organs	Prostate	Weak
	Seminiferous tubules	Weak
	Cervixgative	negative
	Endometrium	Weak
	Fallopian tube	Strong
	Ovary	negative
Central nervous System	Neurons	Strong
Endocrine organs	Thyroid gland	Strong
	Parathyroid gland	negative
	Adrenal Gland	moderate
Immune System	Thymus	Negative
	Lymph nodes	Negative
	Splenic red pulp	Moderate

Figure 3: Expression of EMAPII/pro-EMAPII in Human Tissues (Murray et al.)

Effect on Endothelial Cells

EMAPII was isolated initially on the basis of its ability to induce tissue factor on endothelial cells. From then on, the effect of EMAPII on endothelial cells was extensively studied. This protein brought about an increase in intracellular Ca+2 levels in endothelial cells which was accompanied by an increase in vWF antigen release and increased P-selectin expression (51). It also enhanced the expression of E-selectin comparable to levels expressed when endothelial cells were exposed to LPS. The enhanced expression of E and P selectin would indicate an increased adhesivity of endothelial cells for polymorphonuclear leukocytes (51). Matrigel implant and neovascularization model studies illustrated the ability of EMAPII to suppress neovessel formation in response to bovine fibroblast growth factor. This cytokine possessed antiangiogenic properties targeting the rapidly growing vascular beds (64-65).High doses of EMAPII caused complete apoptosis of endothelial cells whereas low EMAPII doses did not bring about a dramatic decrease in endothelial cell viability. EMAPII induced endothelial cell apoptosis was mediated by Fas associated death domain (FADD) protein up regulation and Bcl2 protein up regulation (65). EMAPII brought about significant gene expression pattern changes in endothelial cells. A total of 63 genes were up regulated and 6 genes were down regulated in the presence of EMAPII; DOC1,ADM, FOS,ICAM1,ID1,ID2,KIT, KLF4, SOCS3,TNFA and IP3 to name a few (66). Apart from being anti angiogenic and apoptotic for endothelial cells, EMAPII is also a chemo attractant (67). Endothelial cells migrate in response to increasing concentrations of EMAPII. The amino acid sequence that occurs at the definition site for chemokines is shared by EMAPII. It shares 50% sequence homology with interleukin-8 within the ten

amino acid long chemokine consensus region (67). Also, EMAPII-induced endothelial cell migration is abrogated by pertussis toxin which blocks G-protein coupled receptor signaling. It also increased intracellular calcium in EPC (67). These observations led to the conclusion that EMAPII belongs to chemokine family. After establishing that EMAPII is a chemokine, the next step was to deduce the receptor employed by this chemokine to induce EC migration.CXCR3 was the ideal candidate owing to its involvement in binding anti angiogenic ligands. Receptor binding competition assays with EMAPII and IP10,a cognate CXCR3 ligand, demonstrated that EMAPII could competitively replace IP10 providing evidence for the use of CXCR3 receptor by EMAPII for signaling in endothelial cells (67).

Effect on leukocytes

EMAPII increased the cytosolic Ca+2 levels and myeloperoxidase activity in PMNs. EMAPII also had an effect on mononuclear phagocyte (MP) populations. In the presence of EMAPII, MPs released TNFα and IL-8 (51). EMAPII is chemotactic for polymorphonuclear cells and mononuclear cells (51).

Recombinant EMAPII is cytotoxic to activated T lymphocytes. EMAPII caused a dose-dependent inhibition of Jurkat cells (activated T cell line) and had no effect on peripheral blood mononuclear cells (55).Recombinant EMAPII inhibits DNA synthesis and cell division in peripheral blood mononuclear cells. Colorectal tumor cell derived soluble and cell surface associated EMAPII also induced apoptosis of Jurkat cells. This apoptosis was associated with the activation of caspase-8 (60).

Regulation of EMAPII

EMAPII is usually expressed in regions that are undergoing programmed cell death and tissue remodeling. MethA fibrosarcoma cells released mature 22Kd EMAPII when they were subjected to apoptosis. This release was not observed when the cells underwent necrosis. EMAPII mRNA expression remained unchanged when Meth A cells were induced to undergo apoptosis. However, as mentioned earlier, EMAPII mRNA co localizes at regions of macrophage accumulation and apoptosis. Thus, apoptosis might be one way in which EMAPII release is regulated in cells. When the cells undergo apoptosis, the precursor form of EMAPII is cleaved in a caspase dependent manner to release the mature EMAPII (62).

Mature EMAPII is also released by tumor cells that were subjected to hypoxia. Hypoxia does not cause an increase in EMAPII mRNA, but causes the release of mature EMAPII. Hypoxic cells also displayed an increased expression of proEMAPII/p43. It was also observed that hypoxic cells that released EMAPII did not undergo apoptosis in parallel since apoptosis was one of the mechanisms that triggered the release of EMAPII. Moreover, release of EMAPII during hypoxia could not be inhibited by caspase inhibitors indicating an alternate pathway for processing proEMAPII during hypoxic conditions (68).Thus, hypoxia and apoptosis are the two main mechanisms by which tumors generate EMAPII.

EMAPII and tumors

When a solid tumor is small, it acquires the necessary oxygen and nutrients via simple diffusion. But when it grows larger, active angiogenesis is required for the supply of nutrients and oxygen. Targeting angiogenesis would therefore be an attractive

proposition for tumor therapy. Inhibitors targeting angiogenic factors have met with reasonable success as anti-tumor agents (69-70). EMAPII possess antiangiogenic properties and induces the apoptosis of endothelial cells. EMAPII also suppresses primary and metastatic tumors (64-65). These properties make EMAPII a viable candidate for tumor therapy. EMAPII was not cytotoxic or anti proliferative to C6 gliomal cells in vitro. However, in vivo EMAPII therapy affected the tumor growth tremendously. Although, EMAPII administration did not abrogate the tumor completely, it led to a tremendous decrease in the tumor volume compared to the control mice that did not receive any EMAPII treatment (71). EMAPII also decreased the tumor proliferative index and brought about vascular thrombosis. The mechanism of action of EMAPII is different from the other antiangiogenic agents used for tumor therapy so far (71).

Systemic infusion of EMAPII in C3H/HeJ and Balb/c mice led to systemic toxicity [transient inflammation] and release of cytokines whereas intratumoral injection led to extensive thrombohemorrhage and increased vascular permeability (51). It also rendered TNF insensitive tumors sensitive to the anti tumor effects of TNF, however this effect was observed only in vivo (51, 72). Contrary to the previously established findings, a prolonged course of rEMAPII treatment did not adversely affect the health of C57BL6/J mice. In the lewis lung carcinoma(LLC) model and human breast carcinoma cell line model, rEMAPII assuaged the growth of tumors without perceptible injury to normal organs (64).Animals injected with high EMAPII expressing melanoma cell lines like 1286 exhibited slower tumor growth and progression compared to those injected with low EMAPII expressing melanoma cell lines like PmeI (73). Injection of retrovirally

transduced PmeI cell line expressing high levels of EMAPII led to slower tumor growth and progression.

Having stated the properties and functions of EMAPII, I would like to shift my focus on another important protein STAT3. STAT3 is also a very important player in tumor immunology. It is therefore important to ascertain whether EMAPII could have any effect on STAT3 expression and functions.

STAT3

Signal transducers and activators of transcription 3 belong to the family of STAT proteins. These are DNA binding proteins which play an important role in dictating the expression of genes. STAT proteins get phosphorylated upon activation. They then leave the cytoplasm as homo- or hetero-dimers and translocate to the nucleus where they activate genes with the specific response elements (74). So far, the STAT family is comprised of seven members (STAT1, 2, 3, 4, 5A, 5B and 6). STAT 1 and 2 were identified based on interferon alpha and gamma dependent activation. STAT3 was identified following activation with epidermal growth factor (EGF) or interleukin-6. It was found that STAT3 could not be activated by interferon gamma (75). G-CSF is another candidate that can bring about the phosphorylation and DNA binding of STAT3 proteins (76).

STAT3 mRNA is highly expressed in brain, liver, kidney, testis, spleen, heart and thymus. Western blot of STAT3 reveals two bands; a 92KD major band and an 83 KD minor band. These might be alternative forms of the same protein. STAT3 shares 30 - 40% sequence identity with other STAT proteins (75). The deletion of STAT3 is embryogenically lethal and it plays pivotal roles in cell growth and division, apoptosis

and maintenance of homeostasis (77-78). It is a major player in controlling innate immunity and regulates NF-κB recruitment (78). Induction of tolerance helps to prevent autoimmune disorders. APCs play a very crucial role in this complex feat of imparting tolerance to self antigens. It has been found that STAT3 signaling is involved in inducing tolerogenic responses from T cells. Increased STAT3 activation in APCs resulted in impaired antigen specific T cell responses and abrogation of STAT3 activation led to autoimmunity. STAT3 seems to be a key player in maintaining the fine balance between tolerance and immune activation (79).

Since STAT3 is associated with several proliferation-associated genes, cell cycle genes and induction of tolerance, it is not surprising for tumor cells to take advantage of the STAT3 pathway to benefit their survival and proliferation. Lassmann et.al found that there is aberrant expression of STAT3 in colorectal cancer cells. Even though the mRNA levels of STAT3 in neoplastic colonic epithelial cells were comparable to the normal epithelial cells, the mRNA levels of STAT3 inducible genes and protein levels of phosphorylated and unphosphorylated STAT3 were significantly higher in colonic cancer cells than in normal epithelial cells. This suggests that the normal regulation of STAT3 activation is compromised in tumor cells without significantly influencing STAT3 mRNA levels (80).Tumor also releases factors that increase the levels of activated STAT3 in myeloid cells. This could alter their differentiation and activation. When the abnormal STAT3 activation in myeloid DCs were inhibited by JSI-124, a selective STAT3 pathway inhibitor, DC differentiated like normal DC and exhibited a dramatic increase in the activation of immature DCs which was evident with an increase in the expression of MHCII, co stimulatory molecules and T cell activation (81).

Summary

Even though EMAPII has been considered as a potential candidate for tumor therapy, the actual role played by this important cytokine in a tumor milieu is not clear. It was first isolated from the tumor supernatant; the actual reason as to why this protein is being released is not understood clearly. One of the prime reasons for this being the dual and contrasting functions exhibited by this protein. Some of its functions are beneficial for the tumor. However, some functions actually boost the immune system and aid in the elimination of the tumor. There could be two possible explanations: one being that it is released by the tumor with an agenda to interfere with the immune system in a way that would allow the tumor to thrive and the second reason could be that it is just a byproduct of another pathway, triggered accidently which now is functioning in favor of the immune system or the tumor.

From the literature, the role played by dendritic cells in tumor immunology is absolutely evident. It will not be surprising to find tumor elaborating factors that could hinder the efficacy of DC. EMAPII could be one such factor released by tumor cells that could alter DC in such a way that would be beneficial for tumor survival. So far, the effects of EMAPII on dendritic cells have not been studied. The current study aims at elucidating the role played by EMAPII in a tumor environment with respect to its effects on dendritic cells. It will help us to understand to some extent whether EMAPII is actually playing for or against the immune system.

As mentioned earlier, the chemotactic roles exerted by EMAPII are evident. The queries posed by the current research could be answered by studying the effect of EMAPII on dendritic cell migration, maturation, release of cytokines, phagocytosis, and pinocytosis.

CHAPTER II

EMAPII- A STIMULANT OF DENDRITIC CELL (DC) MIGRATION

Introduction

Being professional antigen presenting cells, the main function of dendritic cells is to constantly scan the environment for potential threats (1). Upon encountering such a target they capture them via phagocytosis or macropinocytosis (15-16). Immature DC are highly efficient in acquiring antigens and processing them(14). The acquired antigens are presented to T cells in the context of MHC molecules. CD8α lymph node resident DC are the key players in activating T cells during an initial infection (82). However, DC residing in the skin, like Langerhans cells, langerin negative dermal DC and langerin positive dermal DC have to migrate out of their resident zones in order to deliver the signals required to activate T cells in the secondary lymphoid organs (23-24). Therefore the migration of DC plays a very important role in immune responses and is regulated by chemotactic factors or chemokines released during an inflammation (83).

Tumors usually evade immunosurveillance by altering important immune system cells. They elaborate myriads of factors that could aid them in evading immune attack. EMAPII is one such factor released by Meth A fibrosarcoma cells (49).EMAPII is a multifunctional cytokine with a multitude of important biological functions. This protein is thought to be released by cells undergoing stress or apoptosis (54). EMAPII is chemotactic for endothelial cells, polymorphonuclear and mononuclear cells

(51, 67). Owing to the fact that EMAPII is chemotactic for monocytes and utilizes the CXCR3 receptor, it is possible that EMAPII could play a role in the migration of dendritic cells since DC share a common progenitor with monocytes and express the CXCR3 receptor.

In the present study, we evaluated whether EMAPII could induce the migration of DC. The effect of tumor derived and recombinant EMAPII on dendritic cells was determined by in vitro, ex-vivo and in vivo methods. This study shows that EMAPII may play a role in the disappearance/ reduction of LC/DC in tumor bearing animals.

Materials and methods

Animals

BALB/c female mice aged six to twelve weeks were used for the study**.**

Cell lines

JAWSII cell line, an immature dendritic cell line derived from p53 growth suppressor gene deficient C57BL/6 mice, was purchased from ATCC (Manassas, VA). The methylcholantherene induced fibrosarcoma (MethA fibrosarcoma) was generously supplied by Dr. Wolfram Samlowski at the University Of Utah College Of Medicine.

Media and Reagents

JAWSII cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum, 0.002 µg/ml penicillin, 0.2 U/ml streptomycin, 2 mM glutamine and granulocyte-macrophage colony stimulating factor (GM-CSF). Polyclonal anti-EMAP II (rabbit IgG) was obtained from Oncogene Research Products (San Diego, CA) and used to neutralize at a final concentration of 1 µg/ml in culture. Rabbit anti-sera raised against

Coxiella burnetii was used as an isotype control for this antibody, a kind gift from Dr. Ed Shaw at Oklahoma State University. Recombinant human EMAPII was obtained from Biosources (Camarillo, CA).Alexa Fluor anti-langerin antibody was obtained from eBioscience (SanDiego, CA).Anti-mouse I-A^d antibody was purchased from Biolegend (SanDiego, CA).

Tumor model

For in vivo passage of the tumor, 5x 10^6 MethA fibrosarcoma cells in phosphate buffered saline were injected intraperitoneally into mice. For in vitro culture of MethA cells, RPMI 1640 supplemented with 10% bovine growth serum, 0.002µg/ml penicillin, 0.02U/ml streptomycin and 2mM glutamine was used and the cells were incubated at 37^{o}C in a CO_2 incubator. In vivo neutralization of EMAPII involved a 100µl injection of a 50µg/ml concentration of anti-EMAPII along with the MethA cells on day zero and a 100µl injection of anti-EMAPII alone on day five.

To analyze the effect of tumor burden on LC density, varying methA cell densities were used for intraperitoneal inoculation.

MethA tumor supernatant

MethA fibrosarcoma cells (1x10^5/ml) were grown in complete RPMI 1640 medium for three days at 37^{o}C in a CO_2 incubator. Following incubation, the cells were centrifuged at 1500 rpm for 9 minutes at 4^{o}C.The pellet was discarded and the supernatant was used for the assays.

In vivo assessment of LC density

Mice were sacrificed and the ears were harvested. Dorsal skin was peeled off from the cartilage using fine forceps. The ear skin was placed dermal face down on PBS-EDTA (0.02%w/v) for 90 minutes at 37^{o}C.The epidermis of each section was peeled using forceps and the sections were fixed in 10% buffered formalin for 10 minutes at room temperature. After washing twice with PBS, sections were blocked with hydrogen peroxide for 15 minutes at RT on a shaker. Sections were washed thrice with PBS tween followed by addition of anti-Ia^{d} antibody (Pharmigen, Santiago, CA) and incubated overnight. After washing, freshly prepared Vectastain ABC reagent from an immunoperoxidase kit (Vector, Burlingame, CA) was added. VIP peroxidase substrate was added to develop the reaction .Sections were washed and mounted in glycerol PBS on a microscope slide. LC were counted with the use of a brightfield microscope to obtain the density. Epidermal sheets from both ears of each mouse were examined and 10 fields per sheet were analyzed. Each experiment involved ears from at least 4 mice and was performed at least twice. The results consist of the mean number of IA^{d} positive cells/mm^{2} plus or minus the standard error of the mean. In some cases, a two-tailed Student t-test was applied. One asterisk denotes $p < 0.05$ and two asterisks denote $p < 0.01$.

In order to assess the effect of EMAPII on in vivo distribution of LC, 1µg recombinant EMAPII in 30µl PBS was injected subcutaneously at the base of the ear. Control animals received an injection of 30µl PBS.

Ex vivo ear skin LC migration assay

LC migration was performed by following a modified procedure described by Schuler and Steinman(6) and enhanced by the addition of chemokine as detailed in the approach by Kellerman et.al (84) . Briefly, mouse ears were removed, washed in 70% ethanol and the dorsal skin removed by using fine forceps. The dorsal sections were placed split side down in 1ml complete RPMI medium for two hours. After incubation, skin sections were moved to fresh RPMI media with or without the tumor supernatant, recombinant EMAPII or polyclonal anti-EMAPII. The sections were incubated at 37^{o}C with 5% CO_2 for 24 hours. Emigrated cells were collected after 24hrs and the sections were transferred to fresh medium followed by a 24 hour incubation period. After incubation, both 24 hour and 48 hour samples of emigrated cells were pooled and the numbers of migrated Langerhans cells were determined based on DC morphology with the aid of a hemacytometer and brightfield microscopy. For staining with Alexa Fluor tagged anti-langerin antibody, the cells were fixed in 10% buffered formalin. After washing the cells with PBS, 60µl of 1:50 diluted anti-langerin antibody was added to the cells. Following incubation in the dark at RT for 45 minutes, the cells were washed four times with PBS. The pellet was dissolved in 100µl PBS. The numbers of langerin positive cells were determined using a Nikon EFD-3fluorescent microscope (Nikon, Tokyo, Japan).

Transmigration Assay

JAWSII cells were trypsinized. After centrifugation, the cells were dispensed in 1000 µl sterile PBS. To test for the effect of EMAPII, 600 µl of RPMI medium with

sodium pyruvate, glutamine, antibiotics and serum was added to the wells of a sterile 24 well plate. For tumor supernatant, the desired percentage was obtained using the above mentioned media as a diluent keeping 600 µl as the total volume. Each treatment was performed in duplicate. Neutralizing polyclonal anti-EMAPII antibody was added with tumor supernatant at a final concentration of 1µg/ml. Rabbit Polyclonal RK13 anti-coxiella II antibody was added in conjunction with tumor supernatant as an isotype control. Recombinant EMAPII was added to obtain the designated concentrations. Transwell inserts [5.0 µm pore size] were placed in each well and cells of the JAWSII cell line (100µl containing $1x10^5$ cells) were added to each insert. After Incubation at 37^oC in a CO_2 incubator for 4 hours, non-migrated cells were removed by washing with PBS and gently scraping with a cotton swab. The inserts were stained with CAMCO's fixative for 10 minutes followed by CAMCO's staining solution for 20 minutes. The stained membranes were observed at 400X using a brightfield microscope and the stained cells were enumerated (at least 15 fields per membrane were counted).

Results

Effect of EMAPII on LC migration

EMAPII, a multi-functional cytokine, is released by many tumor cells (50-51). This cytokine is chemotactic for cells of the macrophage/monocytes lineage suggesting a possible role in attracting dendritic cells as well (51). We investigated whether tumor derived EMAPII could mediate the migration of dendritic cells. Langerhans cells that are of myeloid origin were used in an ex vivo migration assay wherein epidermis from murine ear was floated on media containing Meth A supernatant with or without anti-EMAPII. Schuler et al and Kellerman et al. have demonstrated that the majority of cells

that migrate out from murine ear epidermis in this model express phenotypic and morphological markers that are specific for Langerhans cells (6, 84). In our system, 58% of the cells that migrated out expressed Langerin, a LC specific marker, detected by immunofluorescence staining. As in Figure 4, epidermal Langerhans cells migrated more in response to 40% MethA tumor supernatant as compared to the medium alone. Anti-EMAPII completely annulled the migratory stimulation exerted by the tumor supernatant. However, the level of migration exhibited by Langerhans cells in response to MethA tumor supernatant blocked with anti-EMAPII was much lower compared to the medium control. A possible explanation to this observation could be that tumor supernatant might contain anti-chemotactic factors and the effect of these factors is more pronounced in the absence of EMAPII. These data indicate a probability of tumor cells elaborating EMAPII in order to attract dendritic cells. The use of recombinant EMAPII would strengthen the data; however, recombinant murine EMAPII is not commercially available. Human EMAPII shares 86% amino acid sequence homology with murine EMAPII (51). We used human recombinant EMAPII to examine a dose response for migration using Langerhans cells. Increasing concentrations of recombinant EMAPII were used in the ex vivo migration assay and the number of Langerhans cells that migrated out were enumerated. As can be observed in figure 5, human recombinant EMAPII induced the migration of Langerhans cells in a dose dependent manner.

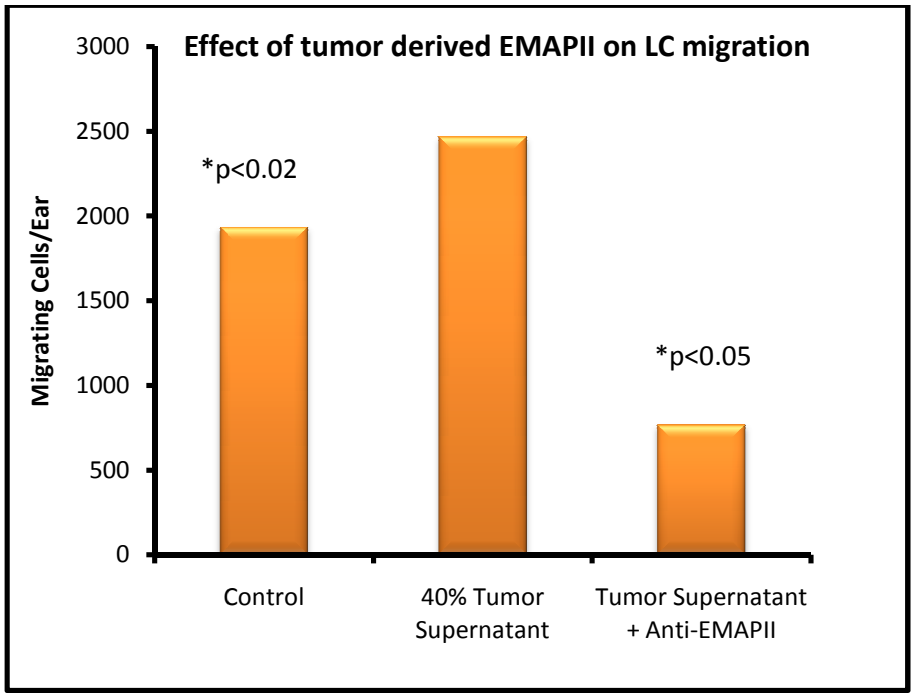
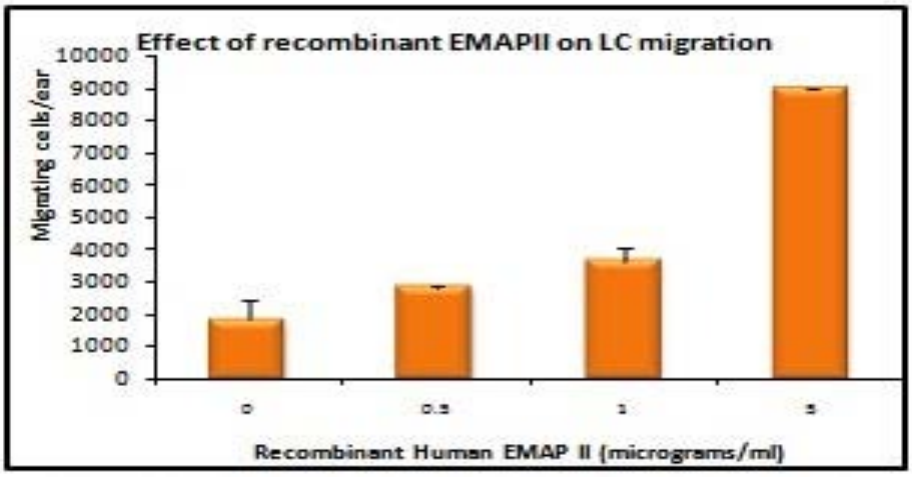


Figure 4: Effect of tumor derived EMAPII on Langerhans cell migration

Dorsal ear skin from Balb/c mice was floated on medium containing Meth A tumor supernatant with or without neutralizing anti-EMAPII (1µg/ml). The cells that migrated out were collected after 24 and 48 hours, pooled and counted. A paired Student t test was employed for statistical analysis.



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Dorsal ear skin from Balb/c mice was floated on medium containing increasing concentrations of human recombinant EMAPII. The cells that emigrated out in

response after 24 and 48 hour incubation periods were combined and enumerated. Error bars depict the standard error of the mean.

In vivo assessment of LC density in tumor bearing animals

Tumor cells release chemotactic factors that attract dendritic cells. These tumor infiltrating dendritic cells (TIDC) have impaired functions in terms of antigen presentation and express molecules that aid in tumor survival (40).We analyzed the density of Langerhans cells in tumor bearing animals. As in figure 6, we observed that the density of Langerhans cells in the murine ear epidermis of tumor bearing animals was much lower as compared to the control animals. In order to ascertain whether EMAPII plays a role in the observed decrease in LC density, anti-EMAPII antibody was injected along with methA fibrosarcoma cells on day zero and day five. Anti-EMAPII antibody treatment partially abrogated the effects of MethA tumor supernatant and the density of LC remained close to that observed in control animals. These data suggest a possible involvement of EMAPII in attracting dendritic cells and this cytokine being a factor released by tumor cells in order to accomplish this. However, anti-EMAPII did not completely reverse the tumor induced decrease in DC density. This could be due to other factors released by tumor cells that could have been repressed by EMAPII, now being able to exert their effects since EMAPII was blocked by its specific antibody. The data is representative of three independent experiments.

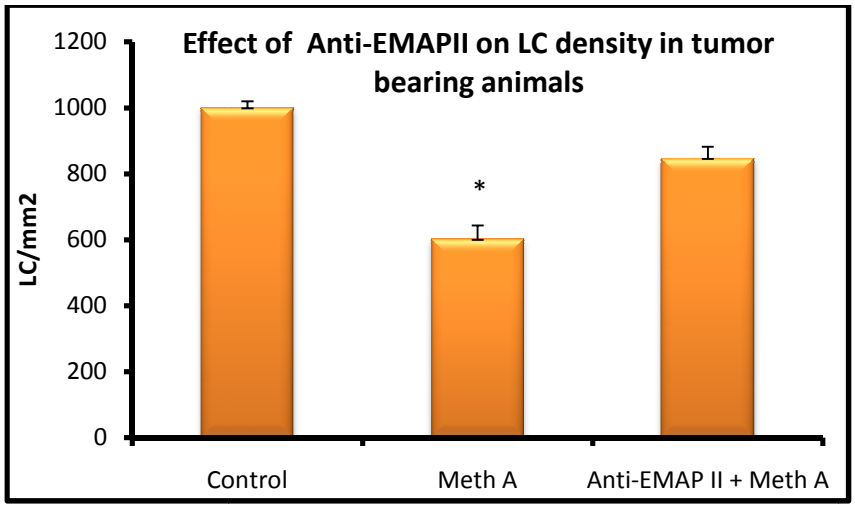


Figure 6: In vivo assessment of LC density

*Balb/c mice were injected with MethA fibrosarcoma cells, 1x10^6 intraperitoneally, with or without anti-EMAPII antibody. Mice received 100µl injections of 50µg/ml injections of anti –EMAPII on day zero and day five. Mice were harvested and the LC density on the ear epidermis was assessed by using IA antibody. The results are representative of three independent experiments. A two-tailed paired Student t test was performed and * indicates a p value< 0.05.*

Effect of EMAPII on the migration of JAWSII cells

We observed that EMAPII could stimulate the migration of Langerhans cells both in vitro and in vivo. In order to assess whether EMAPII could be effective on other DC as well, JAWSII, an immature dendritic cell line of myeloid origin, was employed in a transmigration assay. JAWSII cells were seeded in the upper chamber and MethA tumor supernatant or recombinant EMAPII was placed in the lower chamber. The cells that emigrated through the membrane was stained and counted. As in figure 7, we observed that MethA supernatant stimulated JAWSII cells to migrate and this effect was rescinded by neutralizing anti-EMAPII antibody. An isotype identical antibody control failed to block the migration stimulated by the tumor supernatant. Recombinant EMAPII exerted similar effects as that of the tumor supernatant. JAWSII cells migrated in response to 0.5 µg/ml EMAPII analogous to that of 40% Meth A tumor supernatant. The data strongly

illustrate the ability of EMAPII to attract the JAWSII dendritic cells.

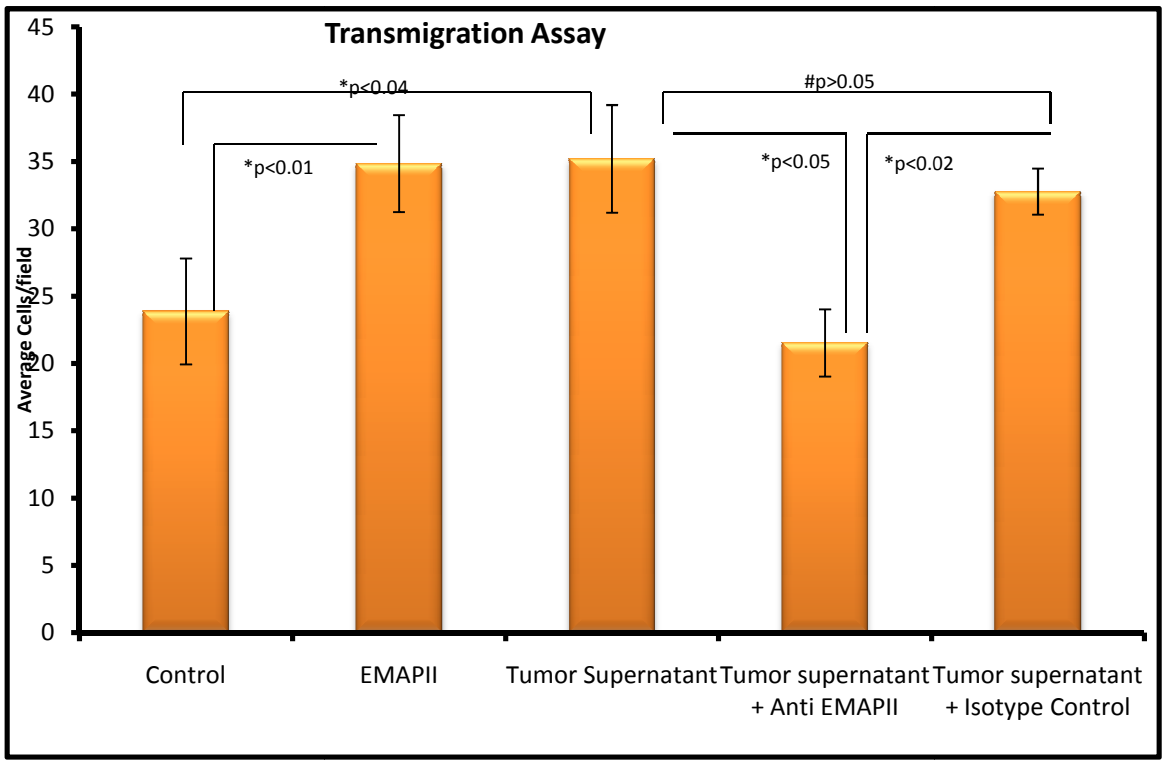


Figure 7: Effect of tumor supernatant and EMAPII on JAWSII cell migration

JAWSII cells at a concentration of 1x10^5 cells in 100 µl were seeded into the upper insert of the transwell insert and complete medium or 0.5µg/ml recombinant EMAPII or 40% MethA tumor supernatant was added at the bottom chamber as stimulant. After 4 hours of incubation, the cells that emigrated through the membrane of the insert were enumerated with the aid of a brightfield microscope. Fifteen fields were counted for each membrane insert. The p values were obtained by means of a paired, two-tailed Student t test.

Effect of recombinant human EMAPII on LC density in vivo

Meth A tumor supernatant seems to be chemotactic for dendritic cells as determined by both in vitro and in vivo methods. Recombinant EMAPII attracted dendritic cells in ex vivo and in vitro assays. We wanted to determine whether the in vitro effects of recombinant EMAPII could be translated in vivo as well. LC density determination assay was performed after injecting EMAPII at the base of the murine ear. Mice were sacrificed after 24 hours and the density of LC in the epidermis was determined by IA staining. As

can be seen in figure 8, injection of EMAPII at the base of the ear stimulated the resident LC to leave the epidermis corroborating the role of EMAPII in LC depletion in tumor bearing mice.

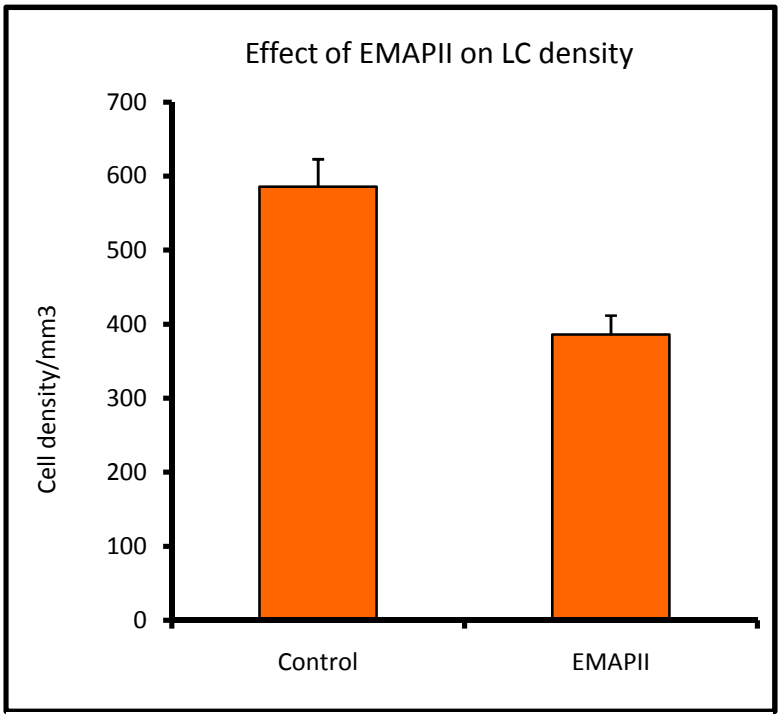


Figure 8: Effect of recombinant EMAPII on epidermal LC density in vivo

Mice were injected at the base of the ear with 1µg recombinant EMAPII or sterile PBS. After 24 hours, ear skin was harvested and the LC density was enumerated after staining the cells with IA specific antibody and immunoperoxidase system.

Discussion

Dendritic cells contribute significantly to anti-tumor immunity. DC are potent stimulators of T cells and can activate tumor specific T cells (51). DC have also been used effectively in tumor immunization (32). However, DC are also actively involved in T cell tolerance induction. They play a crucial role in central as well as peripheral T cell tolerance (85-88). DC can present antigens derived from normal somatic cell turn over to T cells and induce tolerance to these self antigens that might not have been represented in

the thymus. Owing to this dual role played by DC, it is not surprising that tumor cells would devise mechanisms that could alter these important immune system cells to benefit tumor survival. Gabrilovich et al and Idoyaga et al have shown that tumor cells affect dendritic cell maturation and function (35, 40). Also, tumor cells attract dendritic cells and these TIDC have impaired functions (40). We observed that Meth A fibrosarcoma cells decreased the density of Langerhans cells in the murine epidermis. However, these effects were limited to intraperitoneal inoculation of Meth A tumor since subcutaneously implanted Meth A tumor did not decrease the LC density in the periphery. This suggests that tumor would exert systemic effects on DC only after the tumor is well established and when the tumor burden is high. This is in conjunction with our observation that LC density decreases with an increase in the MethA tumor density at the time of inoculation (data not shown). The decrease in LC density could be attributed to multiple factors. A role of apoptosis could be ruled out since we have proven that neither MethA tumor supernatant nor EMAPII could bring about the apoptosis of dendritic cells in vitro. It could be argued that the reduction in LC density is due to the decrease in the influx of LC precursors necessary to populate the epidermis. However, this argument cannot hold true due to the existence of resident LC precursors in the skin. There is also a possibility that LC are leaving the epidermis and are migrating to the lymph nodes or to the tumor.

As mentioned earlier, tumors elaborate factors that could affect dendritic cell maturation and functions and the majority of tumors may attract dendritic cells due to their possible role in T cell tolerance. EMAPII has been shown to be secreted by Meth A fibrosarcoma cells (50). This multi-functional cytokine is chemotactic for monocytes and we wanted to determine whether EMAPII is a factor released by methA fibrosarcoma

cells to attract dendritic cells. We hypothesized that EMAPII could be chemotactic for dendritic cells based on the fact that most dendritic cells share the same myeloid lineage of monocytes and that EMAPII utilizes CXCR3 receptor which is one of the receptors utilized by dendritic cells for chemotaxis (67, 89-90). Our data demonstrates a clear role of both tumor derived and recombinant EMAPII in the decrease in epidermal LC density. The final destination of the skin resident LC is not clear and needs to be determined. In our in vitro studies, we demonstrated that the chemotactic effects of Meth A supernatant could be extended to other myeloid derived dendritic cells like JAWSII cells.

The fact that only 58% of the cells that migrated out from the skin explants was langerin positive could be attributed to the fact that LC that migrate out from skin explants display a mature phenotype and langerin expression is down regulated in mature Langerhans cells (91-92). An alternate explanation could be that EMAPII is chemotactic for both epidermal and dermal dendritic cells since the cells migrated out exhibited typical dendritic cell morphology and IA expression. The ability of recombinant EMAPII to reduce the LC density when injected at the base of the ear provides indisputable evidence supporting the chemotactic role played by EMAPII in dendritic cell migration. According to our study, this multi-functional cytokine can stimulate the migration of dendritic cells belonging to the myeloid lineage and it is one of the factors employed by Meth A fibrosarcoma cells to attract dendritic cells. The fact that anti-EMAPII could not completely reverse the tumor induced decrease in DC density in the periphery suggests that tumor cells are elaborating factors anti-chemotactic that are repressed in the presence of EMAPII.

However, it is uncertain whether the dendritic cells are attracted to the tumor or they are routed to the nearest draining lymph node. Further studies need to be conducted to determine the effects of this cytokine in terms of dendritic cell maturation and functions.

CHAPTER III

EFFECT OF EMAPII ON DENDRITIC CELL FUNCTIONS

Introduction

Dendritic cells play a very important role in the induction of peripheral tolerance (93-94). When cells undergo apoptosis, auto antigens become chemotactic and attract immune system cells like dendritic cells to clear up the debris without evoking an immune response thereby preventing autoimmunity (95). The dendritic cells that reach these sites are tolerogenic in nature and thus aid in peripheral tolerance. EMAPII, a multifunctional cytokine first isolated from the supernatant of MethA fibrosarcoma cells, is released upon apoptosis. EMAPII is chemotactic for dendritic cells, as shown in chapter II, suggesting a possible role played by this cytokine in peripheral tolerance. The mechanisms in place for preventing autoimmunity can often be exploited especially in the case of tumors. Tumors can manipulate the mechanism of tolerance induction to benefit their survival. EMAPII could be one such factor released by cells undergoing apoptosis to alter dendritic cell functions, and in a tumor environment EMAPII could be working in favor of tumor survival by preventing an auto immune response. In order to ascertain this, it is important to determine whether EMAPII can alter DC functions to render them more tolerogenic.

Materials and Methods

Animals

BALB/c female mice aged six to twelve weeks were used for the study.

Cells and Cell lines

JAWSII cell line, an immature dendritic cell line derived from p53 growth suppressor gene deficient C57BL/6 mice, was purchased from ATCC (Manassa, VA). The methylcholantherene induced fibrosarcoma (MethA fibrosarcoma) was generously supplied by Dr. Wolfram Samlowski at the University of Utah College of Medicine. *Saccharomyces cerevisiae* PJ69-4A was a kind gift from Dr.Jeff Hadwiger (Oklahoma State University).

Generation of bone marrow derived dendritic cells (BMDC)

BMDC from Balb/c mice were harvested by using a protocol described by Wong et al. and Matheu et al. with modifications (96-97). Briefly, after sacrificing the mice by carbon dioxide asphyxiation, the femurs were obtained. The bones were cleaned and placed in 75% ethanol for ten minutes. The bones were then transferred to RPMI1640 medium supplemented with 5% fetal bovine serum, 0.002 µg/ml penicillin, 0.2 U/ml streptomycin, 2 mM glutamine. Using a 1ml 26G3/8 syringe, bone marrow was forced out of the bones by flushing the femur four or five times with the media into a 20 X 15mm petriplate containing 15 ml medium. Cells were disrupted by pipetting to obtain single cell suspension. The cell suspension was transferred to a 50 ml centrifuge tube, the petriplates were washed with 10ml medium three times and the suspension was centrifuged at 1500 rpm at 4^{o}C for nine minutes. The supernatant was discarded followed by RBC lysis. For lysing RBCs, 900 µl sterile distilled water was added to the cells.

Within few seconds, 100 µl sterile PBS and 1ml RPMI medium was added. The cells were centrifuged again and the pellet was resuspended in four milliliters RPMI 1640 medium with 10 ng /ml GM-CSF [Primary DC medium].Cells were plated at $1x10^6$ cells/ml in primary DC medium and incubated at 37^oC in a CO_2 incubator. On day four, after removal of the medium, 10 ml fresh primary DC medium was added to the cells. On day seven, cells in suspension were collected, centrifuged, resuspended in 1ml primary DC medium and returned to the original plates with adherent DC. Fresh DC medium was added to the plates followed by incubation at 37^oC in a CO_2 incubator. Cells were harvested on day 10 and used for further analysis and experiments.

Media and Reagents

JAWSII cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum, 0.002 µg/ml penicillin, 0.2 U/ml streptomycin, 2 mM glutamine and 5-10 ng/ml granulocyte-macrophage colony stimulating factor(GM-CSF). MethA fibrosarcoma cells were cultured in RPMI 1640 supplemented with 10% bovine growth serum, 0.002 µg/ml penicillin, 0.02 U/ml streptomycin, 2 mM glutamine. Yeast cells were grown in YEPD media: 10 g yeast extract, 20 g dextrose (glucose) or 40 ml 50% glucose solution, 20 g Bacto-Peptone (Difco) and 1 liter nanopure H_2O. Anti-mouse VEGF antibody was purchased from R&D Systems (Minneapolis, MN).Neutral red solution used for pinocytosis was bought from SIGMA (USA).

Viability assay

JAWSII cells at a density of $1x10^5$/ml were incubated at 37^oC in the presence of MethA tumor supernatant for 24 hours. Cells treated with cyclohexamide were used as the positive control. Following incubation, the plates were centrifuged at 500 rpm for five

minutes. After removal of the medium, 100 µl of MTT [3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (5 mg/ml stock) diluted in complete RPMI medium (1:5 dilution) was added to the wells. The plates were incubated at 37^0C for six hours. The plates were centrifuged at 500 rpm for five minutes. The fluid from the wells was aspirated out and 100 µl isopropanol was added to each well. The purple formazan crystals were dissolved by pipetting vigorously 15 times. The plates were read at 630 nm in the EL-800 microplate reader (Biotek instruments, VT, USA). The absorbance in the medium blank wells was subtracted from each absorbance reading.

Pinocytosis Assay

A modification of pinocytosis assay described by Weeks et.al (98)was followed. JAWSII ($1x10^6$ /ml) cells were incubated on a shaker platform at 20±2°C in JAWSII growth medium with or without EMAPII (0.5 µg/ml), with or without 50% methA tumor supernatant, and 50 µg neutral red for two hours. Serum –free (SF) methA tumor supernatant was also assayed. Each treatment was performed in duplicate. After incubation, cells were centrifuged and the pellet was washed in 5ml Phosphate buffered saline (PBS) at 200 x g for five minutes. The pellet was resuspended in 2.5ml acid alcohol (3% HCL in 95% ethanol). After centrifugation to clear debris, the supernatant was diluted with complete RPMI 1640 medium. The percent transmittance at 533nm was determined using a Schimadzu UV-VIS scanning spectrophotometer (TX, USA). RPMI 1640 medium with 50% acid alcohol was used as the blank for the transmittance measurements. A standard curve was plotted using different neutral red concentrations and the amount of neutral red pinocytosed was derived from the standard curve and

corrected for cell numbers (total µg neutral red per 10^6 cells). Refer to Appendix 2 for calculations and standard curve.

Phagocytosis Assay

Yeast suspension

Saccharomyces cerevisiae PJ69-4A was grown in PYED broth in a shaker at RT. The yeast cells were pelleted and counted using the trypan blue dye exclusion method. To $1x10^8$ yeast cells, 3ml of 0.87 %(w/v) congo red was added. The suspension was allowed to stand for five minutes, 7 ml distilled water was added and the solution was autoclaved for 20 minutes. The yeast cells were then washed with sterile PBS, resuspended in sterile PBS and counted using a hemacytometer.

JAWSII /BMDC cell treatment

JAWSII cells or BMDC at a density of 2.5 x 10^5/ml were incubated with or without EMAPII (0.5µg/ml) in primary DC medium for 24 hours in 24 well plates. Untreated cells were used as control.

Assay

A modification of phagocytic assay proposed by Kaminski et.al (99)and Walsh et al. was followed (100). Briefly, 100 µl of stained yeast suspension ($1.5x10^7$ cells) was added to the treated and control JAWSII cells or BMDC. The set-up was incubated at 37^0C for 90 minutes. Following incubation, the wells were rinsed with sterile PBS. After addition of 1ml sterile PBS, the cells were observed under a brightfield microscope at 400X. JAWSII/BMDC from random fields were assessed (a total of 100) and the numbers of yeast cells phagocytosed by the cells were counted.

Cytokine profiling

Analysis of cytokine transcripts

RNA Extraction

BMDC were cultured at a concentration of $1x10^6$ cells/ml in primary DC growth media with or without recombinant EMAPII (0.5µg/ml) at 37^{o}C in a 5% CO_2 incubator for four hours. A total of three biological replicates for control and treatment groups were set up. Following incubation, the cells were collected by using a cell scraper, centrifuged briefly to pellet the cells and RNA was extracted from the cells using Qiagen's RNeasy kit. RNA was eluted from the spin filter using RNAse free water. After obtaining the concentration and purity of the RNA sample using a NanoDrop ND-1000(s) spectrophotometer (WI, USA), RNA samples were treated with Deoxyribonuclease I (Invitrogen, CA,USA). Briefly, 0.5µg RNA was treated with 1µl DNase buffer and 1µl DNase for 15 minutes at R.T. DNAse was then inactivated by heating at 65^{o}C for ten minutes in the presence of 25mM EDTA.

cDNA Synthesis

DNase treated samples were used for cDNA synthesis with Qiagen's Quantitect Reverse Transcription kit following the manufacturer's protocol. Briefly, 0.5 µg DNase treated RNA samples were incubated with genomic DNA wipeout buffer for two minutes at 42^{o}C followed by incubation with reverse transcriptase master mix at 42^{o}C for 30 minutes. The enzyme was inactivated by incubating the reaction mixture at 95^{o}C for three

minutes. An MJ Research PTC220 thermocycler (Biorad, CA,USA) was used for the reaction.

Cytokine Array

SABiosciences RT^2Profiler PCR Array system was used to analyze the expression of 84 different genes after EMAPII treatment. Briefly, cDNA synthesized by using the above mentioned protocol was diluted with DNase free water to a total volume of 102 µl. SABiosciences RT^2qPCR 2X master mix was added to the diluted cDNA sample and the total volume was made up to 2550 µl. The experimental cocktail [25 µl] was dispensed in all 96 wells of the PCR array plate, the plate was sealed and centrifuged at 500 rpm for 15 minutes. The PCR array plate was run in an Eppendorf Mastercycler using the following cycling conditions: 95^{o}C for 10 minutes, 95^{o}C for 15 seconds, 60^{o}C for 1 minute with a final infinite hold at 4^{o}C . The results obtained were uploaded onto SABiosciences Web portal software in order to calculate the fold regulation and statistics. Ct value is the cycle at which fluorescence crosses the set threshold value. The Ct values of each gene of interest (GOI) were standardized against Glucoronidase B by subtracting the Average Ct of Glucoronidase B from the average Ct of GOI to obtain ΔCt. ΔΔCt was then calculated by subtracting the ΔCt of control/untreated sample from the ΔCt of EMAPII treated sample. Fold change was obtained by $2^{-\Delta\Delta Ct}$ method.

T Cell Chemotaxis Assay

JAWSII cells were grown in the presence of sterile PBS or 0.5 µg/ml recombinant EMAPII for 24 hours at 37^{o}C. Following incubation, the cells were centrifuged and the supernatant was collected. Supernatant form both treated and untreated JAWSII cells were blocked with neutralizing anti-EMAPII antibody for thirty minutes at 37^{o}C. Jurkat T

cells (a kind gift from Dr. Gurunadh Chichli, OMRF) were seeded in transwell inserts (5 µm pore size) and the supernatant from JAWSII cells, used as the chemoattractant, was loaded in the bottom chamber. T cells were allowed to migrate for four hours at 37^{o}C. All the cells that migrated to the bottom well were collected, centrifuged and enumerated using a hemacytometer.

IL-10 Assay

Mouse IL-10 Quantikine immunoassay kit (R&D Systems, USA) was used to assess IL-10 released by JAWSII cells. JAWSII cells, 2x10^{5}, were incubated with or without 5 µg LPS/ 5 µg recombinant EMAPII for 48 hours. The cells were centrifuged at 1500 rpm for nine minutes. The supernatant was collected and used for the assay.

Briefly, 50 µl of the supernatant was added to quantikine ELISA plates with the assay diluent. Mouse IL-10 kit control was used as the positive control and calibrator diluent was used as the negative control. After two hours incubation, the wells of the ELISA plate was washed five times followed by addition of 100 µl of IL-10 conjugate. The plate was incubated for two hours. Following incubation and washing, 100 µl substrate was added to the wells and incubated in the dark. Stop solution was added after 30 minutes and the optical density was measured using EL-800 microplate reader (Biotek instruments) at 450nm.

Proteome Array

Proteome Profiler^{TM} Mouse Cytokine Array panel [R&D Systems, MN, USA] was used to analyze the expression of 40 different analytes following EMAPII treatment. JAWSII cells at a concentration of 1.2X10^{6} was incubated in JAWSII growth medium with or without recombinant EMAPII [1.73 µg final concentration] for 48 hours at 37^{o}C

in a CO_2 incubator. After the period of incubation, cells and the medium were centrifuged for 20 seconds and the supernatant was collected and used for the assay. The assay was carried out following the manufacturer's protocol. Briefly, 600 µl of the sample was incubated with buffer 4, buffer 5 and detection antibody cocktail from the array kit at 20±2^{o}C for one hour. The mixture was added to blocked nitrocellulose arrays and incubated overnight on a shaker platform at 4^{o}C.Following subsequent washes and incubation with Streptavidin HRP, the nitrocellulose membrane was developed using chemiluminescent detection substrate (USBiological, Massachusetts, USA). An AlphaInnotech HP Imager (AlphaInnotech, CA, USA)was used to develop the blots. Data was analyzed using the Image J software (NIH).

STAT3 Expression and Activation

Western Blot

Sample Preparation

JAWSII cells at a concentration of 1x10^{6}/ml were subjected to GM-CSF starvation by growing the cells in JAWSII growth medium without GM-CSF for 24 hours. The cells were then incubated in the presence or absence of 1 µg EMAPII or PBS or 1 µg LPS for four hours at 37^{o}C in a CO_2 incubator. Lysates from JAWSII/BMDC treated with 1µg LPS and from HeLa cells treated with INF-α were used as positive controls. After incubation, the cells were scraped using a cell scraper, centrifuged at 12000 rpm for 20 seconds. To the pellet, 100 µl RIPA lysis buffer with sodium metavanadate , sodium azide, PMSF, benzaminidine and aminocaproic acid was added. The cell suspension was incubated at 4^{o}C for 30 minutes on a shaker platform. The suspension was centrifuged, the supernatant was carefully collected, stored at -20^{o}C and used for the assay. Protein concentration of the lysates were determined by using a

Bradford Assay(101). Briefly, 0.8 ml 1:4 diluted Bradford reagent was mixed with 2 µl of the cell lysate. The suspension was mixed thoroughly by vortexing and incubated at 20±2^{o}C for two minutes. A blank consisted of 2 µl lysis buffer and 0.8 ml diluted Bradford reagent. Optical density at 600nm was determined using Schimadzu UV-VIS scanning spectrophotometer (TX, USA). Protein concentration was determined by using the following formula:

Protein Concentration in µg/µl = Absorbance at 600nm X 10

PAGE and Western Blot

A reducing PAGE gel with 5% stacking and 10 % resolving gel was used for the separation of proteins. Kaleidoscope Precision Plus marker (Biorad, USA) was used as the protein ladder. 40 µg of the protein lysate was loaded and the gel was run at 120 volts. After separation, the proteins were blotted onto a nitrocellulose sheet. Blotting was done overnight at 4^{o}C at 30 Volts. After transfer, the nitrocellulose membrane was stained with 0.1% amido black solution in 10% Glacial Acetic Acid for one minute on a rotating shaker and destained at 20±2^{o}C for 20 minutes. The blot was then photographed UVP GelDoc It Ts Imaging System (Upland, CA). Following destaining, the blot was blocked in blocking buffer for 1 hour at 20±2^{o}C followed by overnight incubation at 4^{o}C. The blot was incubated in 1:2000 diluted rabbit polyclonal anti-mouse STAT3 phospho Y705 antibody (Abcam,USA) at 4^{o}C overnight. The blot was washed four times for ten minutes each with wash buffer. 1:2000 diluted anti-rabbit IgG horse radish peroxidase was used as the secondary antibody. The blot was incubated with secondary antibody for one hour at 20±2^{o}C. Following incubation, the blot was washed four times with wash

buffer. The blot was developed by adding 2 ml chemiluminescent substrate (USBiological, Massachusetts), USA) for horse radishperoxidase enzyme and exposing to AplhaInnotech HP imager.

Cell Surface Marker Expression

Flow Cytometry

Sample Preparation

BMDC were seeded at a concentration of $1.2X10^6$ in complete growth medium with or without 5 µg/ml LPS and/or 1 or 2 µg/ml recombinant EMAPII. Untreated control cells received sterile PBS instead of EMAPII or LPS. The cells were incubated at 37^oC in a 5% CO_2 incubator for 48 hours. Following incubation, the cells were scraped using a cell scraper and centrifuged at 10,000 rpm for 5 seconds. Pelleted cells were fixed by incubating with 10% buffered formalin for ten minutes. The cells were washed with PBS and resuspended in 500 µl of filter sterilized PBS-sodium Azide-Bovine Growth Serum (BGS) solution. The cells of each treatment were stained for CD80, CD86 and I-A expression. Cells were double stained for I-A and CD80. To the 500µl cell suspension of treated and untreated cells, 12µl of 1:100 diluted Fluorescein Isothiocyanate (FITC) conjugated anti-mouse MHCII (I-A/I-E) antibody(eBioscience, SanDiego, CA) and 30 µl of 1:100 diluted Phycoerythrin (PE) conjugated anti-mouse CD80 antibody (eBioscience, SanDieigo,)was added to obtain a final antibody concentration of 0.06 µg/ml. For staining CD86, 12 µl of 1:100 diluted anti-mouse CD86 FITC conjugated antibody was added to the cell suspension separately. Isotype controls for the antibodies were purchased from eBioscience and included PE Armenian Hamster IgG isotype control and FITC Rat IgG2b isotype control. The cells were incubated with the antibodies at 20 ± 2^oC in the dark on ice for 30 minutes. Cell suspension was then slowly layered

over 1 ml bovine growth serum (BGS) and centrifuged at 1500 rpm for five minutes. The pellet was dissolved in 500 µl PBS-Sodium Azide-BGS solution and analyzed by using a Becton Dickinson FACSCalibur flow cytometer (NJ, USA).

Results

Viability Assay

The release of mediators that would bring about the apoptosis of important immune system cells actively involved in tumor immunity would be beneficial for the tumor. The effect of Meth A fibrosarcoma tumor supernatant on the viability of JAWSII cells was assessed using the Method of Transcriptional and Translational assay (MTT). The assay is based on the ability of live cells to reduce MTT to purple formazan crystals. Tumor supernatant at concentrations of 50% and 80% were used for the analysis. Tumor supernatant was tested initially as a primary screen. If tumor supernatant induced JAWSII cell death, EMAPII would have been tested to ascertain whether the tumor releases EMAPII to induce DC cell death. Cyclohexamide [a potent protein inhibitor] was used as a positive control for cell death. The results are summarized in Figure 9. MethA tumor supernatant at 50% and 80% concentrations did not affect the viability of JAWSII cells. Treatment with cyclohexamide completely abrogated growth and viability of JAWSII cells. Compared to the medium alone control, the OD of 50% and 80% MethA supernatant treatment , 0.791 and 0.701 respectively, did not significantly differ implying that the factors released by MethA tumor supernatant did not adversely affect the viability of DC. There is a possibility that tumor supernatant contains growth stimulating factors that is suppressing the effects of the growth inhibitors.

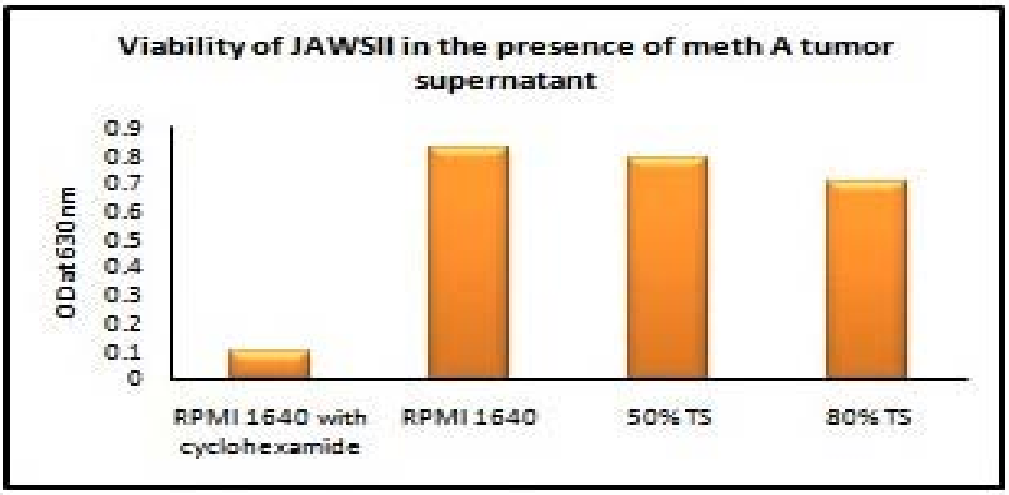


Figure 9: Viability Assay

JAWSII cells (1x10^5/ml) were incubated with methA tumor supernatant for 24 hours. The treated cells were then incubated with MTT for six hours. Cyclohexamide was used as positive control for cell death. The OD of purple formazan crystals solubilized in isopropanol after the six hour incubation period was determined at 630nm using a microplate reader.

Effect of EMAPII on DC Pinocytosis

Dendritic cells are potent antigen presenting cells. They take up antigens by micropinocytosis, macropinocytosis and phagocytosis (15-16). Some of these antigens are acquired by receptor mediated endocytosis as well. Since pinocytosis is one of the classic functions of dendritic cells, it is important to determine whether EMAPII affects the ability of DC to pinocytose. A modification of pinocytosis protocol described by Weeks et.al was followed. In order to eliminate the possibility of serum proteins affecting the pinocytic ability of DC, supernatant from MethA cells grown in serum-free RPMI 1640 medium was also used as a control. JAWSII cells in the presence of 50% MethA tumor supernatant had reduced pinocytic abilities as compared to control cells incubated in the absence of tumor supernatant as evidenced in figure 10. From the graph, it is clear

that serum proteins did not influence the pinocytosis of JAWSII cells. Since, MethA tumor supernatant reduced the pinocytosis of DC; the experiment was repeated with recombinant EMAPII to ascertain whether EMAPII was the agent present in the tumor supernatant that caused a reduction in pinocytosis. EMAPII did not abrogate the pinocytosis of JAWSII cells (Figure 11). Even though, the neutral red accumulated by EMAPII treated JAWSII cells were lower compared to the control cells, there was no was no significant reduction and the effect was not as pronounced as tumor supernatant treatment. This data suggest that EMAPII does not directly affect the pinocytic abilities of dendritic cells and it is not the factor employed by tumor cells to reduce the pinocytic abilities of dendritic cells.

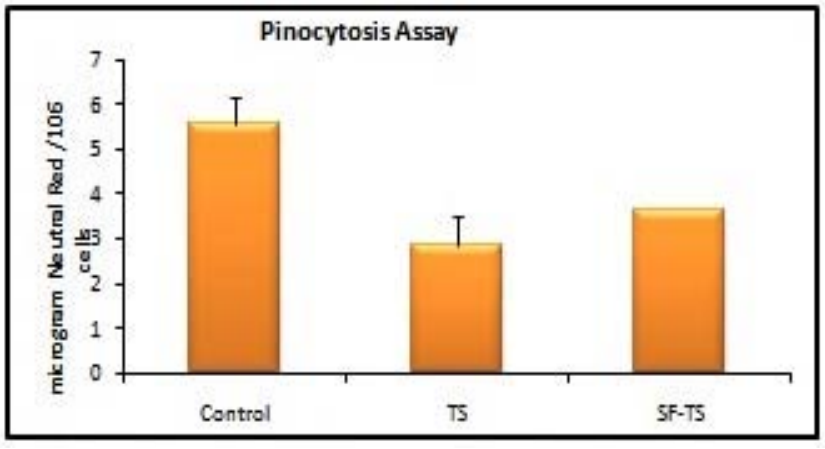


Figure 10: Effect of MethA supernatant on JAWSII Pinocytosis

JAWSII cells at a concentration of $1x10^6$ /ml were incubated in the presence of 50% Meth A supernatant with or without serum and 50 µg neutral red for 2 hours. Following incubation, the pellet was resuspended in acid alcohol and the debris was cleared by centrifugation. The supernatant obtained was diluted in complete RPMI1640 medium and the percent transmittance of the solution at 533nm was determined using UV-VIS scanning spectrophotometer and was used to calculate the amount of neutral red taken up by 10^6 JAWSII cells.

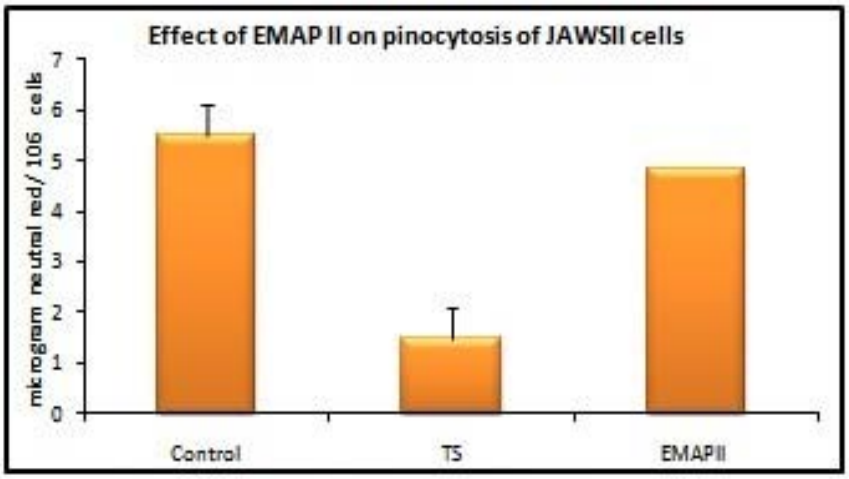


Figure 11: Effect of EMAPII on JAWSII pinocytosis

The effect of EMAPII on JAWSII pinocytosis was assessed by incubating1x10^6 /ml JAWSII cells in the presence of 0.5 µg/ml EMAPII and 50 µg neutral red for two hours and measuring neutral red intake. The cells were washed with acid alcohol and centrifuged and the supernatant was diluted with complete RPMI 1640 medium. The transmittance at 533nm was measured and was used to calculate the amount of neutral red taken up by 10^6 JAWSII cells.

Effect of EMAPII on DC phagocytosis

Dendritic Cells, being professional antigen presenting cells, capture and process antigens in order to present them to T cells or to engage other immune system cells. Phagocytosis is an endocytic process by which cells like DC capture large antigens through an actin dependent mechanism (102). JAWSII cells and BMDC were both tested for their ability phagocytize after treatment with EMAPII for 24 and 48 hours. The number of yeast cells phagocytized by each phagocyte was enumerated and the phagocytic index was calculated (Figure 12, 13). Phagocytic index is the average number of cells engulfed per antigen presenting cell/phagocyte. The percentage of DC that

exhibited more than 5 yeast cells within the cytoplasm was also assessed (Figure 14, 15). EMAPII treated DC (JAWSII and BMDC) exhibited low phagocytic indices compared to the untreated Control cells. The number of yeast cells that could be phagocytosed by DC was also reduced when the cells were treated with EMAPII. This was evidenced in figure 14 and 15 where the percent of DC that captured more than 5 yeast cells was very low compared to the untreated controls. EMAPII treatment however did not completely abrogate the ability of DC to phagocytize.

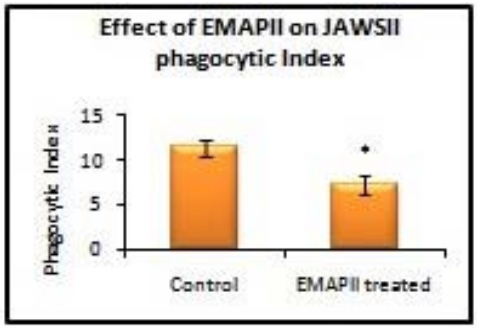


Figure 12: The effect of EMAPII on BMDC Phagocytic Index

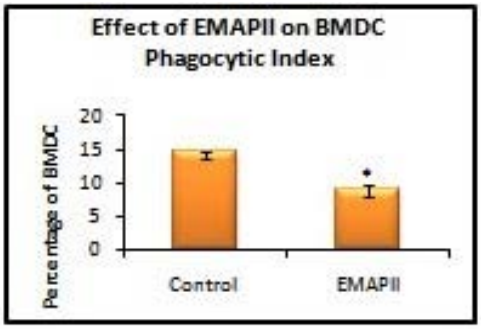


Figure 13: The effect of EMAPII on JAWSII Phagocytic Index

*The JAWSII cells/BMDC (2.5x10^5) were treated with EMAPII for 24 hours ,the treated cells were incubated with heat killed stained yeast suspension and the phagocytic index was determined by counting 100 dendritic cells after 90 minutes using a brightfield microscope. The figure is a representative of three/ four identical experiments. * p=0.0075 for the JAWSII trial and 0.0158 for BMDC trial.*

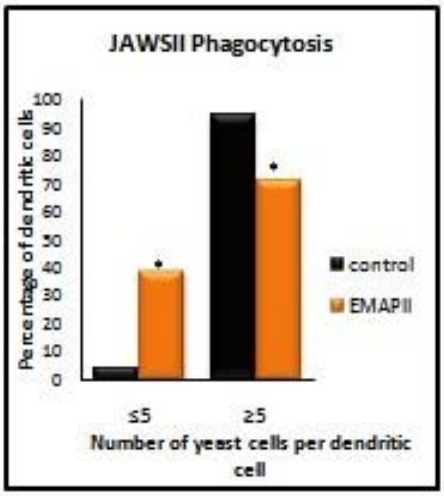


Figure 15: The effect of EMAPII on JAWSII Phagocytosis

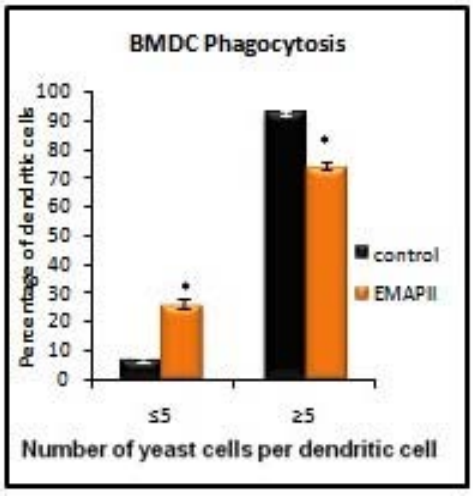


Figure 14: The effect of EMAPII on BMDC Phagocytosis

*JAWSII cells/BMDC (2.5x10^5/ml) were treated with EMAPII for 24 hours, the treated cells were incubated with heat killed stained yeast suspension and the number of yeast cells phagocytosed by JAWSII and BMDC after EMAPII treatment was enumerated. The percentage of dendritic cells that engulfed more than five yeast cells was determined. The graph is a representative of three identical experiments. *p=0.0043 for BMDC trials and 0.0018 for JAWSII trial. A paired Student T test was used for statistical analysis.*

The reduction in DC phagocytic index in the presence of EMAPII indicates that EMAPII interferes with the phagocytic pathway probably giving tumor cells an advantage by reducing the chances of tumor associated antigens to be phagocytosed effectively by dendritic cells. Also, the observation that the percentage of DC that take up less than five yeast cells when treated with EMAPII suggests that EMAPII is down regulating receptors that are required for effective uptake of the antigens.

Cytokine Profiling

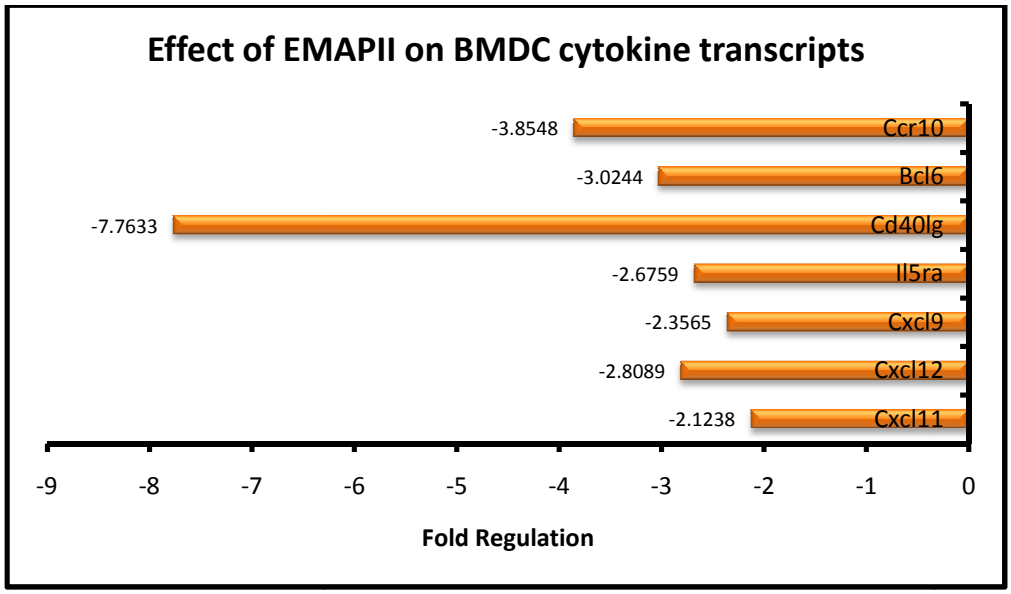
As antigen presenting cells, dendritic cells are the key players to activate naïve T cells. T cells mature upon receiving maturation signals which could be the cytokines released by

the APC upon their interaction with a T cell and this interaction is mediated by chemokines. DC are capable of releasing myriads of cytokines and based on the surrounding environment, some cytokines are up regulated and some are down regulated. Based on the cytokines they are exposed to, T cells can become regulatory T cells or follow a Th1 or Th2 lineage. The effect of EMAPII on dendritic cell cytokine profile was assessed to determine whether tumor elaborates this cytokine to affect the DC in ways that would alter their normal cytokine function which would abrogate the functions of cells involved in anti-tumor immunity. Cytokine transcripts in DC with or without EMAPII treatment was determined by using a Cytokine profiler kit which could test for 84 different cytokine transcripts and five housekeeping genes. GusB or beta Glucoronidase was used for normalizing the data since some of the other housekeeping genes like actin were up regulated after EMAPII treatment. Interestingly, the cytokines that were affected by EMAPII treatment that exhibited statistically significant fold changes were all down regulated. Figure 16 tabulates the fold regulation of cytokine gene expression after EMAPII treatment and the average delta Ct values of the genes that showed statistically significant change in gene expression is graphed in Figure 17. Ct value or threshold cycle is the cycle at which fluorescence crosses the set threshold value. Delta Ct is the difference between the Ct values of the gene of interest and the house keeping gene (in our case GusB). Average delta Ct is the average of the delta Ct obtained from three biological replicates.

Genes	Fold regulation	Genes	Fold regulation	Genes	Fold regulation
Abcf1	5.6699	Ccr8	2.8024	Il1f6	-2.1287
Bcl6	-3.0244	Ccr9	-2.555	Il1f8	-2.0658
Cxcr5	-2.9759	Crp	1.3287	Il1r1	-2.3674
C3	-3.7755	Cx3cl1	5.1934	Il1r2	-2.1685
Casp1	-2.2815	Cxcl1	1.4241	Il20	-2.1386
Ccl1	-2.2553	Cxcl10	-2.0515	Il2rb	-1.203
Ccl11	-1.8877	Cxcl11	-2.1238	Il2rg	-2.8812
Ccl12	-1.0968	Cxcl12	-2.8089	Il3	-1.544
Ccl17	-1.9679	Cxcl13	-1.3379	Il4	-2.3403
Ccl19	-1.7736	Cxcl15	-2.7766	Il5ra	-2.6759
Ccl2	-3.7581	Pf4	-3.2868	Il6ra	1.0892
Ccl20	1.162	Cxcl5	-3.9816	Il6st	1.0892
Ccl22	14.2874	Cxcl9	-2.3565	Il8rb	-3.5636
Ccl24	2.6208	Cxcr3	-1.217	Itgam	-2.6574
Ccl25	-1.4241	Ccr10	-3.8548	Itgb2	-2.682
Ccl3	5.3765	Ifng	-2.9966	Lta	-2.9214
Ccl4	-3.2565	Il10	-1.5404	Ltb	-3.2868
Ccl5	-2.2658	Il10ra	-2.3241	Mif	-2.4566
Ccl6	-2.7766	Il10rb	-2.6268	Scye1	-2.4967
Ccl7	1.4675	Il11	-1.9725	Spp1	1.1147
Ccl8	-3.0596	Il13	-1.7736	Tgfb1	-3.234
Ccl9	-2.3784	Il13ra1	-2.835	Tnf	-1.9908
Ccr1	-2.5432	Il15	-3.1456	Tnfrsf1a	-1.651
Ccr2	-2.2449	Il16	-3.249	Tnfrsf1b	-3.7064
Ccr3	-1.8067	Il17b	-2.4284	Cd40lg	-7.7633
Ccr4	-2.2553	Il18	-2.3027	Tollip	-2.5967
Ccr5	-2.6574	Il1a	-2.3134	Xcr1	-2.7702
Ccr6	-2.3403	Il1b	-2.4967	Ccr7	-1.4241

Figure 16: Fold Regulation of Cytokine transcripts expressed by BMDC after EMAPII treatment

BMDC (1x10 ^{6}cells/ml) were incubated in the presence of 0.5 µg/ml EMAPII for four hours. Following RNA extraction, a cDNA synthesis kit was used to reverse transcribe mRNA to cDNA. cDNA was mixed with the SABiosciences RT^{2} qPCR mastermix and dispensed in the PCR array plate. After 40 cycles, delta Ct values and fold change values were calculated using the Web portal software available through the manufacturer's website. A fold change of 2 and above was considered a significant change in gene expression.



Fold regulation of the genes that showed statistically significant expression changes and a twofold regulation after EMAPII treatment when compared to untreated control is plotted. A Student t test was employed and a p value of 0.05 and below was considered statistically significant.

T cell Chemotaxis Assay

Down regulation of CXCL9, CXCL11 and CXCL12 transcripts would decrease the chemotaxis of T cells in response to dendritic cells. In order to determine whether the results obtained at transcript levels could be translated physiologically as well, a T cell migration assay in response to dendritic cells treated with EMAPII was performed. JAWSII cells were incubated in the presence or absence of EMAPII for 24 hours and the supernatant obtained from the cultured cells analyzed for their ability to attract T cells. Since EMAPII is apoptotic for lymphocytes or might have other direct effects on T cells, the supernatant was treated with neutralizing anti-EMAPII antibody for 30 minutes to neutralize EMAPII present in the supernatant. Supernatant from both untreated control and EMAPII treated cells were neutralized before the chemotaxis assay. As can be seen in figure 18, supernatant from DC treated with EMAPII showed a significant reduction in

its ability to attract T cells. The probability of EMAPII affecting the viability of T cells being a reason for this reduction can be ruled out since EMAPII was neutralized using a specific antibody. This again would be advantageous to the tumors since blocking chemotaxis of T cells would enhance the chances of tumor survival and proliferation. This corroborates our earlier finding that EMAPII treatment reduces chemokines like CXCL9, CXCL11 and CXCL12 that are chemotactic for T cells.

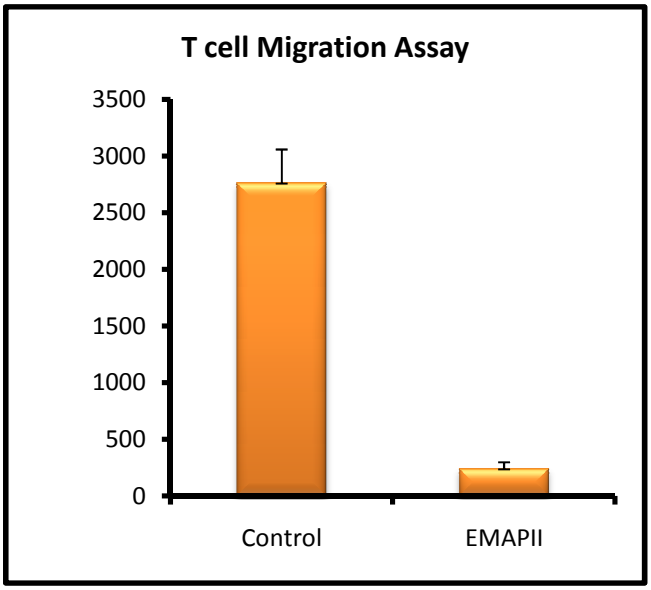


Figure 18: T cell transmigration assay

JAWSII cells ($1x10^6$/ml) were incubated in the presence or absence of EMAPII for 24 hours. The supernatant from these cells were assayed for their ability to attract T cells in transmigration assay. T cells ($1x10^5$ in 100 µl) were seeded in the top chamber and the supernatant was loaded in the bottom chamber. The number of cells that migrated to the bottom chamber was enumerated after four hours by using a hemacytometer and a brightfield microscope. This is a representative of three independently conducted experiments.

IL-10 Assay

IL-10 exerts several effects on APC such as macrophages, monocytes and dendritic cells. IL-10 abrogates MHCII, CD86 and CD58 expression on dendritic cells. It renders the DC ineffective in terms of inducing T cell responses of primed and naïve T

cells. In addition, IL-10 treated DC induced an anergic response from $CD4^{+}$T cells (103). The ability of EMAPII to induce the release of IL-10 from dendritic cells was assessed by performing an ELISA to detect picogram quantities of IL-10 released after EMAPII treatment. As in figure 19, Meth A supernatant and LPS induced the release of IL10. However, EMAPII treatment did not induce IL10 production. In conjunction with LPS or Meth A supernatant, EMAPII augmented the release of IL10 in response to these agents. But this was not a very significant change. One of the reasons why EMAPII could increase the release of IL10 in response to the tumor supernatant could be that EMAPII is antagonistic to some of the IL10 inhibitors thereby suppressing their effects to a greater extent.

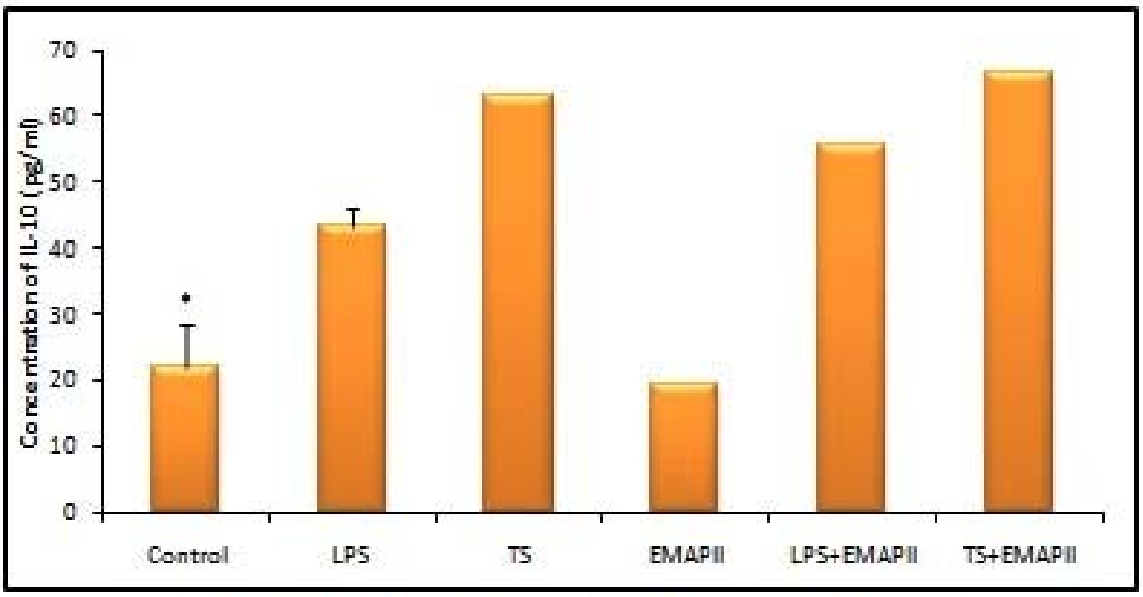


Figure 19: Quantification of IL-10 release by ELISA

IL-10 release from JAWSII cells was assessed by an ELISA based method wherein 2x10 ^{5} JAWSII cells/ml were incubated in the presence or absence of EMAPII and/or LPS/ MethA tumor supernatant for 48 hours and the supernatant was assayed for IL-10. indicates a p value >0.05.*

Proteome Array

The profile of cytokines released by dendritic cells play an important role in shaping the immune response. We wanted to determine whether treatment with EMAPII changed the cytokine profile of dendritic cells. At transcript levels, we observed that EMAPII down regulated the expression of many important cytokines. To ascertain whether this effect is translated at protein levels as well, we used a proteome profiler array to measure the cytokine secretion after EMAPII treatment and compare with untreated controls. JAWSII were employed for the purpose. As can be seen in figure 20, EMAPII treatment changed the cytokine profile of JAWSII cells. The representative of two experiments is shown in figure 20. EMAPII treatment lowered the cytokine levels of JAWSII cells. I309, eotaxin, IL1 beta, Il3, IL5, IL7, IL12, M-CSF and MIP-1 alpha were some of the cytokines whose

expression was significantly down regulated. The levels of these cytokines were less than 50% in comparison to the controls. It is interesting to note that the cytokines that were down regulated by EMAPII were all pro inflammatory cytokines there by assuaging the chances of an anti tumor immunity to develop.

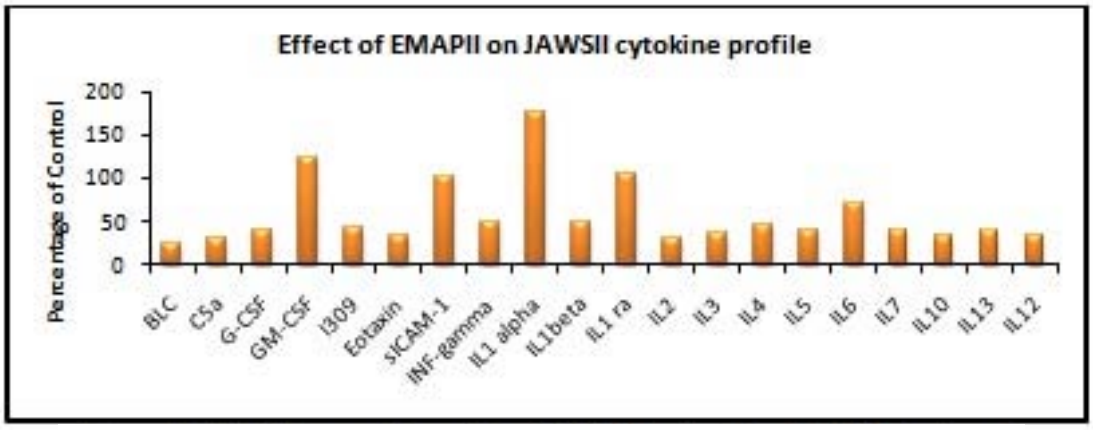


Figure 20: Cytokine protein profile of JAWSII cells after EMAPII treatment

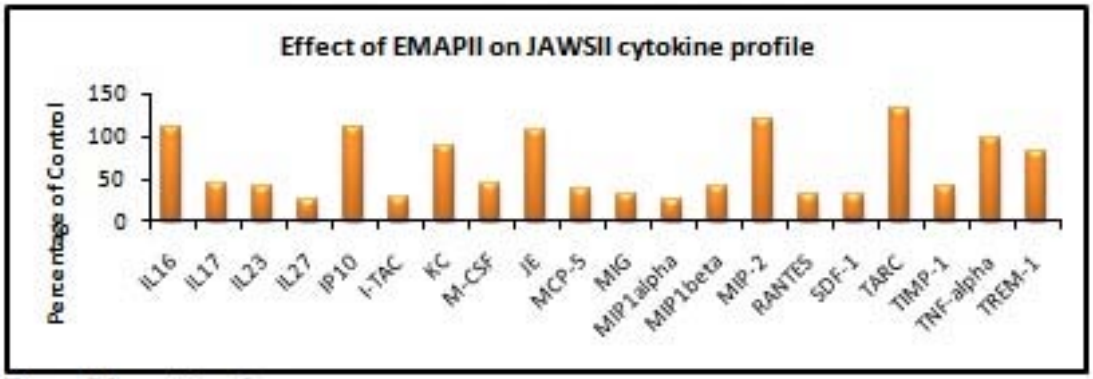


Figure 20 *continued*....

JAWSII cells at a concentration of 1.2x10^6 /ml were incubated in the presence or absence of EMAPII for 48 hours. The cells were centrifuged and the supernatant was used for the analysis. Nitrocellulose membranes blotted with antibodies against the 40 analytes to be tested was incubated with the supernatant. The blot was developed using a chemiluminescent substrate. Chemiluminescent signals obtained from the blots were measured using the Image J software and the integrated pixel densities were obtained. Normalization was done by multiplying the integrated pixel density values by a factor obtained by comparing the average pixel densities of the positive control spots on the EMAPII treated and untreated

blots. The pixel densities of individual cytokines on EMAPII treated blots were divided by the pixel densities of the control spots. The results are reported as percentage of control.

Cell Surface Marker Expression

In order to activate T cells, the antigens have to be presented in the context of MHCI or MHCII molecules by an antigen presenting cell along with additional co-stimulatory signals through CD80 and CD86 (104). Abrogating the ability of DC to activate T cells will clearly give tumor cells an added advantage since they can evade T cell mediated anti-tumor responses. To ascertain whether EMAPII can down regulate MHC expression by dendritic cells, BMDC were incubated in the presence or absence of EMAPII for 48 hours. Cells were incubated with FITC conjugated anti-mouse I-A or anti-mouse CD86 antibody and PE conjugated anti-mouse CD80 antibody. Cells were double stained for CD80 and I-A. After 30 minutes incubation on ice and after washing, the cells were resuspended in PBS-Sodium azide-BGS solution and analyzed by flow cytometry for MHCII/Ia [murine counterpart], CD80 and CD86 expression. EMAPII did not up regulate or down regulate IA/ CD80 or CD86 expression on JAWSII or BMDC. The expression levels of these surface molecules were comparable to that of the untreated control (Figures 21-24). EMAPII did not up regulate the cell surface maker expression on dendritic cells implying that it cannot bring about DC maturation like its precursor protein pro-EMAPII. However, EMAPII did not down regulate the expression of cell surface molecules either thereby suggesting that it does not block DC maturation by blocking the signaling through costimulatory molecules and/or MHCII molecules.

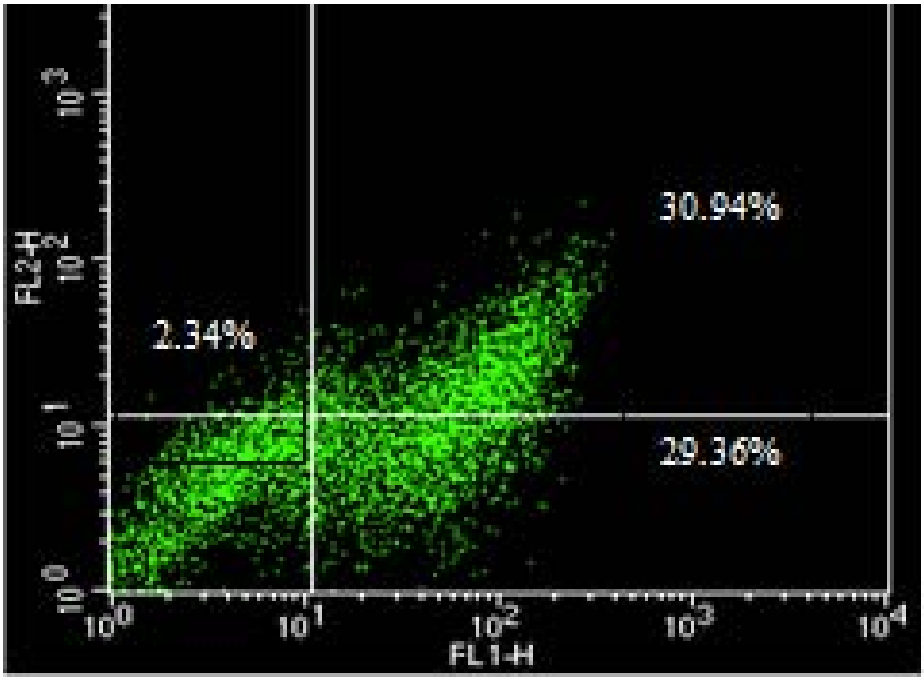


Figure 21: The cell surface expression of Ia and CD80 on untreated BMDC

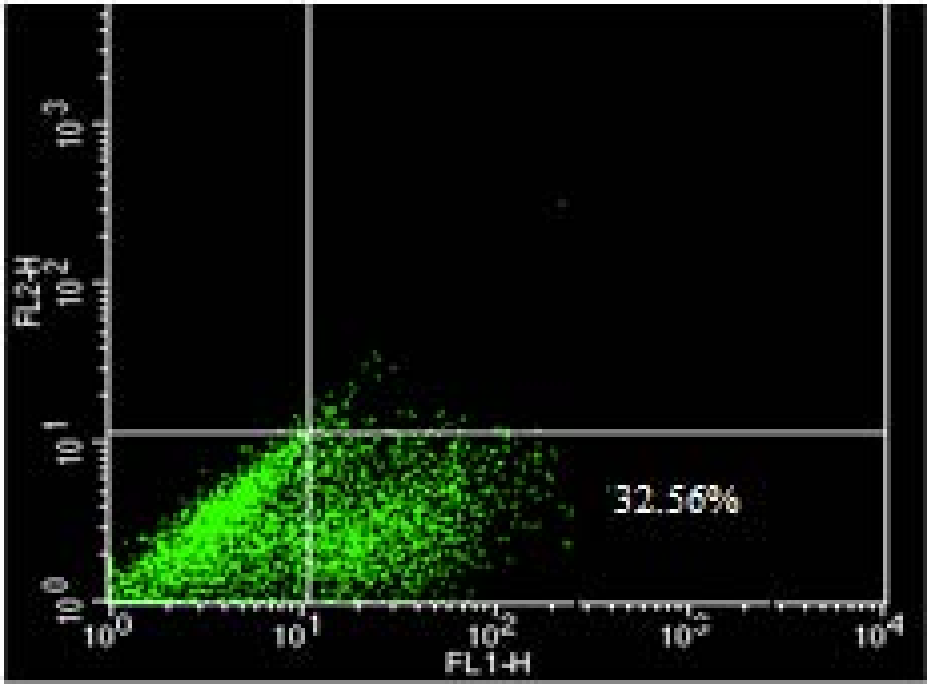


Figure 22: The cell surface expression of CD86 on untreated BMDC

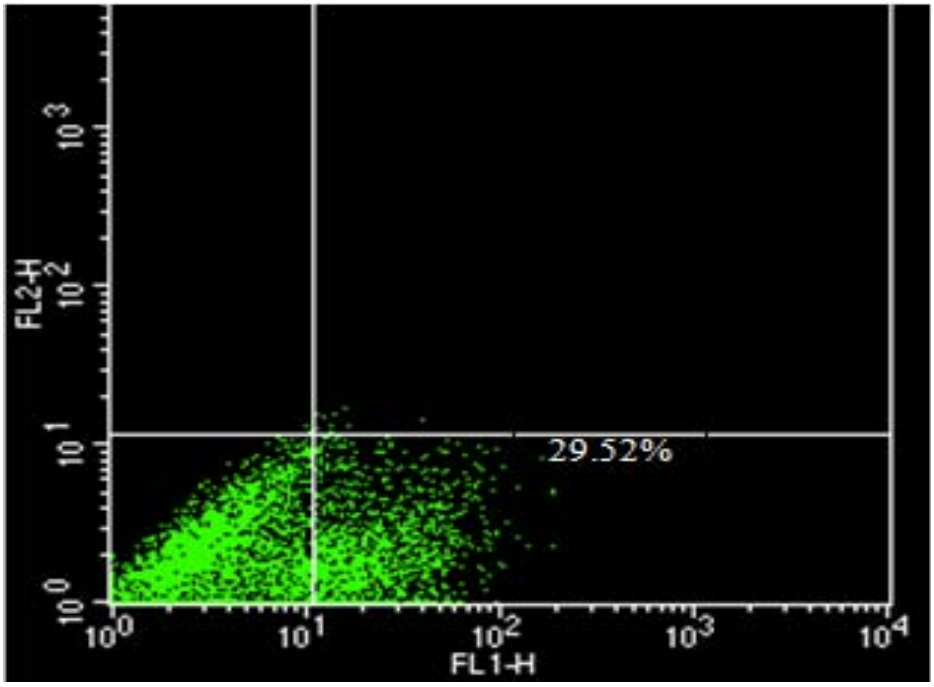


Figure 23: The Cell surface expression of CD86 on BMDC treated with EMAPII

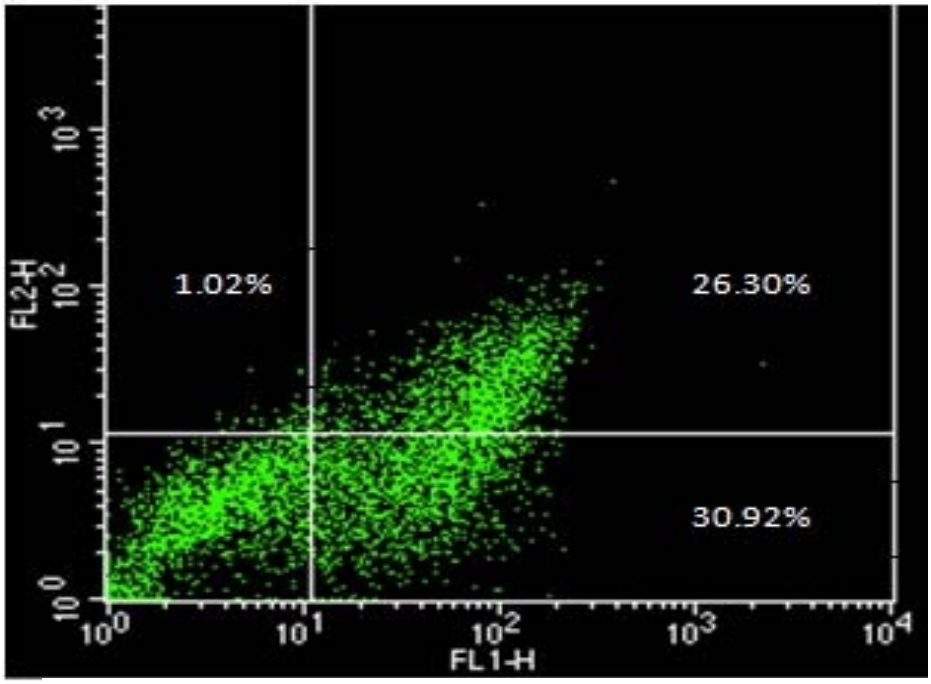
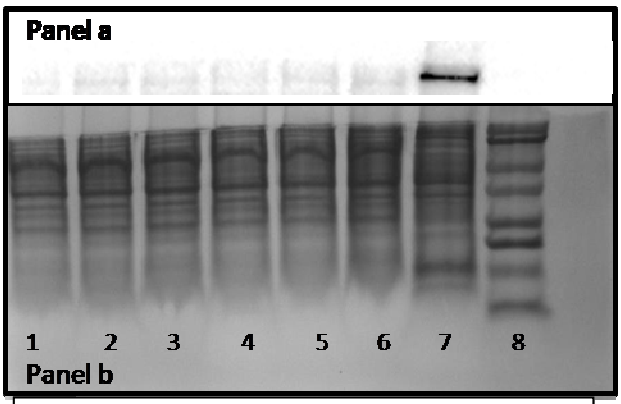


Figure 24: The cell surface expression of Ia and CD80 on BMDC treated with EMAPII

BMDC ($1.2x10^6$ /ml) were incubated with EMAPII for 48 hours and the expression of IA, CD80 and CD86 were analyzed by flow cytometry using FITC or PE conjugated antibodies against IA, CD80 or CD86. In figure 23 and 24, the fluorescence intensity on the X axis corresponds to the CD86 expression. In figure 21 and 22, the number of IA positive cells (X axis) is depicted on the lower right quadrant and the CD80 positive cells(Y axis) are on the upper left quadrant. The double positive cells are in the upper right quadrant. The cell surface expression was based on fluorescence intensity.

Stat3 Expression

It has been documented that STAT3 can suppress the activation /maturation of dendritic cells (81, 105). Tumors elaborate factors that induce hyperactivation of JAK2/STAT3 pathway that leads to abnormal DC differentiation. We wanted to investigate whether EMAPII is one of the factors released by tumor cells to block the differentiation or activation of DC by activating the JAK2/STAT3 pathway. JAWSII cells were incubated in the presence of EMAPII or LPS for four hours and the level of activated STAT3 protein was assessed by Western Blot. STAT3 is a transcription activator that affects dendritic cell functions (78, 81, 105). STAT3 is activated by phosphorylation of its tyrosine or serine residues. As shown in figure 25, the levels of phosphorylated STAT3 in EMAPII treated DC was comparable to the untreated DC control cells. LPS treatment completely failed to activate STAT3. The level of phosphorylated STAT3 was quite low in all treated and untreated DC when compared to the HeLa cell lysate that was used as a positive control. These results suggest that EMAPII does not employ and activate STAT3 and EMAPII is not the tumor derived factor that causes an up regulation of STAT3 activation in tumor bearing animals.



1 LPS treated JAWSII cell lysate (replicate 1)
2 EMAPII treated JAWSII cell lysate (replicate 1)
3 Untreated JAWSII cell lysate (replicate 1)
4 LPS treated JAWSII cell lysate (replicate 2)
5 EMAPII treated JAWSII cell lysate (replicate 2)
6 Untreated JAWSII cell lysate (replicate 2)
7 HeLa cell lysate (positive control)
8 Kaleidoscope Precision Plus Marker

25: Analysis of STAT3 activation by
n Blot

Panel a: JAWSII cells (1x10^6/ml) were incubated with EMAPII, following GM-CSF starvation, for four hours. The cells were lysed using an RIPA based lysis buffer with anti-phosphatases. Protein concentration was determined using Bradford assay and equal amounts of protein was loaded and run on a reducing PAGE gel. The separated proteins were blotted on nitrocellulose sheets and equal loading was ascertained by amido black staining. STAT3 phosphorylation was detected by using anti-mouse STAT3 phospho Y705 antibody and the blot was developed by using chemiluminescent substrate. The results are representative of more than three independent experiments
Panel b: Total protein stain using amido black to ascertain equal loading.

Discussion

Cancers represent a heterogeneous multicellular complex of cells and soluble factors that interact dynamically to favor uncontrolled proliferation and differentiation. This complex interaction involves many players including the tumor cells themselves, soluble factors and mediators released by different cell types that represent the

microenvironment, for e.g., fibroblasts, endothelial cells, and the cells of the acquired and innate immune response. In order to survive and proliferate, tumor cells elaborate myriads of factors that could alter the immune system cells. An important immune system cell that plays a vital role in anti-tumor immunity is dendritic cells (29-30, 32, 106). It has been reported that DC from tumor bearing animals or tumor infiltrating DC are functionally compromised (107). There are numerous ways in which tumors can incapacitate dendritic cells. One of the strategies employed by tumor cells to keep immune system cells under check is via induction of apoptosis. Tumor cells release factors like EMAPII which can bring about lymphocyte apoptosis (55, 60). Therefore, we analyzed the ability of EMAPII to affect the viability of dendritic cells in vitro. We demonstrated that Meth A supernatant does not interfere with DC viability and this observation is in conjunction with the findings that most tumors are infiltrated with dendritic cells suggesting a possible role of these DC in aiding tumor survival (108). This observation is in line with the fact that DC orchestrates myriads of immune reactions and are important in eliciting regulatory T cell functions and therefore might not be subjected to apoptosis by tumor cells that can gain advantage by exploiting the tolerogenic functions of DC (109). At this point it can be safely concluded that the tumor takes advantage of the chemotactic properties of EMAPII rather than the apoptotic properties with respect to dendritic cells.

Dendritic cells are professional antigen presenting cells that coordinate many immune system functions including activation of T cells. Being antigen presenting cells they are equipped with a variety of antigen acquiring mechanisms namely receptor mediated endocytosis, macropinocytosis, micropinocytosis and phagocytosis (1). We

wanted to determine whether EMAPII could alter the pinocytic abilities of dendritic cells. Recombinant EMAPII failed to affect the pinocytic abilities of dendritic cells; however MethA tumor supernatant treatment greatly reduced the pinocytic index of dendritic cells suggesting a possibility of factors other than EMAPII playing a role in DC pinocytic functions. One possible factor could be tumor-derived VEGF since blocking tumor supernatant with neutralizing anti-VEGF antibody slightly reversed the reduction in pinocytosis induced by MethA tumor supernatant [data not shown].

Phagocytosis is an energy dependent , receptor-mediated phenomenon employed by antigen presenting cells to engulf particulate antigens or organisms (1). One of the mechanisms employed by tumors to render DC inactive is by inhibiting the phagocytosis and antigen processing capacity of dendritic cells (108). Here, we formally demonstrated that EMAPII treatment reduced the phagocytic index of both bone-marrow derived dendritic cells and JAWSII cells. This observation corroborates the finding that dendritic cells in a tumor milieu have reduced phagocytic capacities. EMAPII did not completely inhibit the phagocytic abilities of dendritic cells; instead we saw a decrease in the phagocytic index in terms of the number of cells engulfed. This could be attributed to EMAPII decreasing the receptors required for phagocytosis. Phagocytosis of *Saccharomyces cerevisiae* by murine dendritic cells involves Dectin-1 receptor since mannose receptor , the other major receptor employed by phagocytic cells to phagocytize yeast cells, is absent in murine dendritic cells (110-111). The reduction in phagocytic index after EMAPII treatment suggests a possibility that EMAPII down regulates Dectin-1 receptor expression on DC cell surface. Though the relevance of Dectin-1 down regulation in our current model of study is quite vague, the role played by Dectin-1

engagement in dendritic cells in converting regulatory T cells to IL17 releasing T cells and cytotoxic T cell priming is worth a mention (112-113). In this context, it is understandable if the tumor cells down regulate receptors that could switch a regulatory T cell to one with proinflammatory functions.

Cytokines are soluble molecules that regulate the responses of immune system cells and control the outcome of an immune response (114). Being antigen presenting cells, dendritic cells are equipped with a wide array of cytokines which they implement in order to coordinate an immune response (1). The cytokine profile of an environment plays a crucial role in determining whether inflammatory responses or regulatory responses would ensue (114). Cytokines can therefore play a very important role in a tumor environment. We assessed the cytokine profile of BMDC after EMAPII treatment. The genes that showed significant expression changes after EMAPII treatment were all down regulated with respect to the non-treated control cells. There was a 7.7 fold down regulation of CD40 ligand. However, since EMAPII treatment did up regulate many cytokine genes, it can be safely concluded that EMAPII did not cause an absolute shut down of all the pathways. These genes did not meet the statistically significant criteria of a p value of 0.05 and below and was not considered for further analysis. The reason why beta actin could not employed as the house keeping gene was owing to the fact that EMAPII up regulated its expression. Therefore, the gene expression changes induced in dendritic cells by EMAPII is selective and not as a result of a complete shutdown of the signal transduction pathways.

CD40 is a transmembrane glycoprotein that was initially thought to be expressed on the surface of cells like dendritic cells, B cells, macrophages/monocytes, endothelial

cells, platelets, smooth muscle cells and fibroblasts (115-117). The ligand specific for CD40, CD154 or CD40L ,is expressed on T lymphocytes, endothelial cells, smooth muscle cells and epithelial cells (118-119). CD40-CD154 interaction provides the essential co stimulatory signals required for T cell activation (118). In terms of B cells, CD40-CD40L interaction promotes antibody production, cytokine release and isotype switching (120). This interaction also improves macrophage effector functions and maximizes priming and memory formation in CD8 T lymphocytes via dendritic cells (115, 118, 121-123). The above mentioned interactions are between CD 40L on T cells and CD40 on antigen presenting cells. However, there is increasing evidence that CD40 ligand is also expressed by dendritic cells and CD 40 is expressed by T lymphocytes (122). CD40 expression on T lymphocytes increased in cases of autoimmunity suggesting that CD40 on T cells promotes autoimmunity due to re-expression of recombinase activating gene (RAG) and T cell receptor rearrangement in $CD40^{+}$ T cells (124-125).Johnson et al. demonstrated that CD8 T cells can be activated in a CD4 independent manner by employing CD154 expressed by dendritic cells (122). According to a study conducted by Wagner et al., CD40 engagement on thymocytes induces RAG-dependent Vα expression suggesting a potential autoimmune trigger (125). Having stated the importance and functions of dendritic cell CD40L-T cell CD40 interaction, it is not surprising for the tumor cells to down regulate the expression of CD40L on dendritic cell surface. CD40 L engagement would potentially work against the tumor due to the ability of this interaction to induce RAG gene re-expression and induction of autoimmunity. This will potentially lead to anti tumor immunity and enhanced immune response against the tumor antigens. We have demonstrated that EMAPII treatment down regulates the

expression of CD40L on dendritic cells up to seven fold. This provides a strong argument favoring EMAPII being released by tumor cells to enhance tumor survival.

CXCL9, CXCL11 and CXCL12 belong to the CXC family of chemokines. These chemokines are potent chemo attractants for T lymphocytes and play an important role in adaptive immunity. CXCL9 and CXCL11 act via the CXCR3 receptor on T lymphocytes whereas CXCL12 utilizes CXCR4 and CXCR7 receptors (126-130). Treatment with EMAPII down regulated the expression of these chemokines suggesting that tumor cells are trying to keep the adaptive immune responses in check by reducing the infiltration of T lymphocytes. Hou et al. demonstrated that EMAPII employs CXCR3 receptors for the chemotaxis of endothelial progenitor cells (67). One of the possible explanation to the observed decrease in the expression in CXCL9 and CXCL11 could be attributed to the fact the release of EMAPII down regulated the chemokines specific for CXCR3 in order to prevent competitive binding. This data strongly favors EMAPII being released in order to benefit tumor survival rather than to facilitate anti tumor immunity.

CCR10 serves as the receptor for CC family of chemokines like CCL27 and CCL28 (131-132) . CCR10 is usually up regulated in skin homing T lymphocytes and CCL27-CCR10 interaction mediate this chemotactic response (133) . The role played by CCR10 in dendritic cells is not very clear. According to Zou et al. CCR10 is up regulated on embryonic stem cell derived dendritic cells in response to LIGHT family of proteins and is involved in the migration of dendritic cells (134). However, conflicting observations are reported by Homey et al. who claims that CCR10 is not expressed in CD34 positive progenitor –derived or monocytes derived dendritic cells (133). We have demonstrated that EMAPII treatment down regulates CCR10 expression however; the significance of

this finding is quite unclear at the moment due to the lack of information regarding CCR10 expression on dendritic cells.

BCL6 is a transcriptional repressor expressed mainly by B cells, dendritic cells and macrophages (135-137). BCL6 is constitutively expressed in immature dendritic cells, however when the cells mature, BCL6 levels are down regulated to allow a window of transcriptional activation (137). It was surprising for EMAPII to down regulate BCL6 since decrease in BCL6 levels usually is accompanied by a mature phenotype. However, while analyzing this data, it has to noted that BCL6 expression patterns in DC changes with respect to different stimuli ranging from no effect to transient down regulation or complete irreversible down regulation (137).

Interleukin 5 is a hematopoietic growth factor that is essential for the differentiation and growth of eosinophils (138) . IL5 also induces the generation of cytotoxic T cells and is actively involved in the proliferation and differentiation of B cells (139-140).EMAPII treatment reduced the expression of IL5 and its receptor subunit IL5 α in dendritic cells rendering them ineffective in terms of aiding in B cell differentiation and T cell generation.

EMAPII treatment substantially reduced the expression and release of inflammatory cytokines like IL1 beta, IL3, IL5, IL7 and IL12. IL1 beta is involved in the proliferation and differentiation of CD4 T cells and mediates T cell independent DC activation. DC activated in the presence of IL1 beta induces stronger IFN gamma responses from T cells and the release of CD40 ligand mediated cytokine release which includes IL12 (141). Since CD40L is expressed by non T cells as well, non T cells could also contribute to the release of CD40 ligand cytokines in the absence of cytokine

signaling from T cells.IL1 beta enhances cytokine release via CD40L pathway in a T cell independent form. Some of the cytokines that are released after CD40L engagement are IL12 and IL6, both detrimental to the tumor (141). EMAPII treatment not only reduced the expression of IL1 beta, it also decreased the CD40L expression on dendritic cells thereby reducing the probability of the alternative pathway being activated in the absence of T cell help. This would eliminate the release of cytokines that would activate T cells or dendritic cells thereby steering the immune response towards a strong anti-tumor response.

IL3 is a myeloid growth factor that can greatly enhance the tumor antigen acquisition and generation of potent tumor specific T cell response (142-143). Dendritic cells treated with EMAPII released lower amounts of IL3 when compared to the untreated control cells. This would again favor tumor survival by lowering the signals that would enhance the activation of cytolytic T cells.

IL7 is yet another proinflammatory cytokine that was down regulated by dendritic cells in response to EMAPII. IL7 is a pleiotropic cytokine with very important roles in T and B cell development (144). It regulates the proliferation and development of T lymphocytes and augments the effector functions of tumor infiltrating lymphocytes (145). Due to its ability to enhance anti tumor immune responses, IL7 has been considered as a potential candidate for tumor therapy (146). Considering the fact that IL7 has been used in tumor therapy, it would be advantageous for the tumor to reduce the expression of IL7. In our model, release of EMAPII reduced the expression of IL7 emphasizing the role played by EMAPII in favor of the tumor.

Release of IL12 is one of the most important steps that mark the maturation of

dendritic cells. IL12 skews the immune response to a Th1 type and majority of the tumors devise mechanisms to down regulate this cytokine due to its potent ability to induce strong cytotoxic responses (147-148). Several strategies could be employed for this and one would be the release of cytokines that would switch the cytokine profiles of dendritic cells and macrophages (149). EMAPII seems to decrease IL12 production from dendritic cells thus enhancing the chances for tumor survival. Even though EMAPII did not induce the release of IL10 by itself, it augmented the release of IL10 from dendritic cells in the presence of LPS and Meth A supernatant. This suggest that EMAPII could, in the presence of other stimuli cause a Th1 to Th2 shift which would be beneficial for the tumor cells.

Some of the other cytokines that were down regulated include MIP-1 alpha and eotaxin, both of which are up regulated in mature dendritic cells. In the presence of EMAPII, DC failed to up regulate these cytokines that are important for inflammatory responses and for attracting lymphocytes and monocytes (150-151).

Dendritic cells are potent professional antigen presenting cells that activate naïve T cells. DC are usually found in an immature state in the tissues where they continually scan the environment for potential threats. They are the sentinel cells of the immune system. Immature DC are characterized by increased antigen acquiring capacity and decreased antigen presenting capacity in terms of reduced expression of co stimulatory molecules, adhesion molecules and MHCII molecules. DC mature when they interact with DC and present their captured antigens to T cells. DC maturation is marked by up regulation of co stimulatory molecules, MHC molecules and adhesion molecules and down regulation of antigen acquisition. Presentation of antigen on MHCI or MHCII

molecule is crucial for T cell activation. The T cell receptor must bind the peptide-MHC complex with high affinity. CD80 and CD86 are the two of the crucial co stimulatory molecules required for DC maturation. They bind to CD28 on T cells (10). This pathway is very complicated and complex due to the involvement of multiple ligands and receptors. Grossi et al. have demonstrated that in the absence of co stimulatory signals through the CD80/CD86-CD28 pathway, T cells cannot get activated, instead they become anergic and tolerance is induced (152). This phenomenon is very crucial for peripheral tolerance toward self antigens. Tumors can take advantage of this DC characteristic. EMAPII did not alter the cell surface expression of Ia (mouse MHCII), CD 80 and CD 86 on dendritic cell surface. This was quite surprising owing to the fact that CD80 and CD86 expression and signaling are enhanced and prolonged through a signal from CD40-CD40L interaction and EMAPII down regulated the CD40L expression on DC (153). However, it can be argued that EMAPII decreased the CD40L expression on DC and not T cells and there is no evidence to indicate that T cell CD40- DC CD40L interaction is required for co stimulatory signals. Our data does not corroborate the findings of Kim et al. who demonstrated that AIMP1 (pro EMAPII) induced the maturation of BMDC with an up regulation of Ia and co stimulatory molecules CD80 and CD86 (58). Partly this discrepancy in findings could be attributed to the fact that we used EMAPII in our study and they have used the precursor of EMAPII, pro-EMAPII/p43. Structurally EMAPII is 22KD and consists of around 166 amino acids; on the other hand proEMAPII is 35KD and is made up of 312 amino acids (56). Even though EMAPII is a cleavage product of AIMP1, Kim et al. have interchangeably used the terms EMAPII and AIMP1 for the 35KD protein they had used in the assays. The up regulation of

proinflammatory cytokines like IL-12 by AIMP1 was not mimicked by EMAPII. This strongly argues against the use of EMAPII for anti-tumor therapy since EMAPII favors tumor survival in terms of altering DC functions.

Aberrant expression or activation of STAT3 is associated with DC dysfunctions in many tumors (80, 105). We speculated that since EMAPII employs CXCR3 in endothelial cells for signaling events, there is a possibility that it would recruit STAT3 protein as the transcriptional activator. EMAPII did not alter STAT3 transcript level (data not shown) or phosphorylated STAT3 proteins suggesting that EMAPII might be employing some other transcription factor other than STAT3 and the alterations of DC functions that are manifested after EMAPII treatment is most likely to be independent of STAT3 pathway. Therefore, the DC dysfunctions that is observed after EMAPII treatment is not due to aberrant STAT3 activation.

From the results obtained from our study we have proposed a model discerning the role played by EMAPII in a tumor milieu. This is illustrated in figure 26. Figure 27 depicts the model for effects of EMAPII as described by Tas et al.(52). We have modified this model to incorporate the effect of dendritic cells as well (figure 28). When tumor cells begin to undergo apoptosis or under hypoxic conditions, pro-EMAPII is cleaved in a caspase dependent or independent manner to release EMAPII. The released EMAPII attracts dendritic cells from the periphery. We speculate that these DC are attracted to the tumor site since the destination of the DC that disappeared after EMAPII treatment is not elucidated. The dendritic cells that home into the tumors will have altered functions since the binding of EMAPII reduces their phagocytic functions which render them ineffective in terms of acquiring tumor associated antigens. This would benefit the

tumor since impaired or reduced phagocytosis can render the dendritic cells tolerogenic. Complete blocking of phagocytosis however will not work in favor of tumor cells since that will abrogate tolerogenic DC from presenting the acquired antigens and thereby inducing tolerance towards tumor antigens. EMAPII binding does not result in dendritic cell maturation therefore will not up regulate the expression of Ia and co stimulatory molecules. Since chemokines like CXCL9,CXCL11 that are released by dendritic cells to attract T cells is down regulated in the presence of EMAPII, the number of T cells migrating to the tumor milieu will be limited. T cells that manage to migrate to the tumor will have a high probability of becoming tolerogenic since autoantigens usually fail to induce maturation of DC which in turn will render the T cells anergic. To overcome suppressive or tolerogenic signals, T cells must receive signals from CD40 ligand interactions and signals from co stimulatory molecules. Though EMAPII does not down regulate co stimulatory molecules, it down regulated CD40L expression on DC indicating that the signals required for strong inflammatory responses was down regulated. Under normal conditions, EMAPII might be released by cells undergoing apoptosis to attract dendritic cells in order to induce peripheral tolerance. Tumors are exploiting this function of EMAPII to induce peripheral tolerance to benefit their survival.

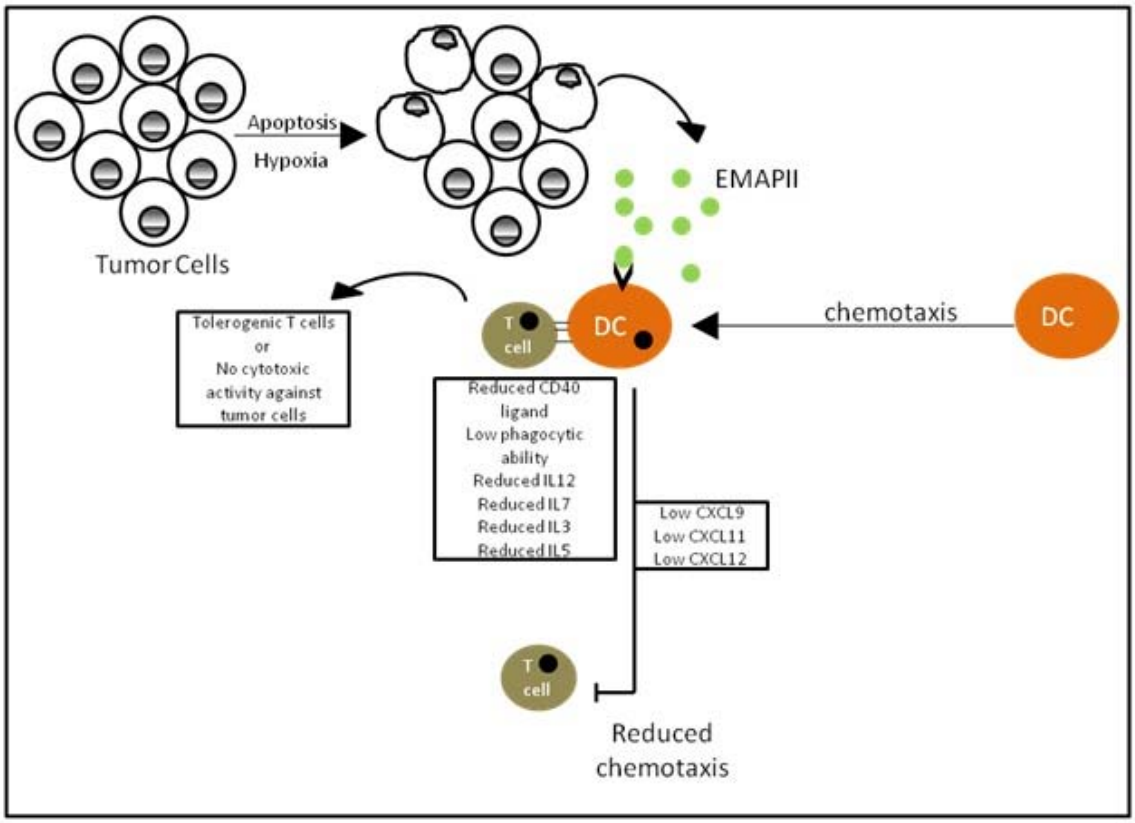


Figure 26: Role of EMAPII in a tumor milieu

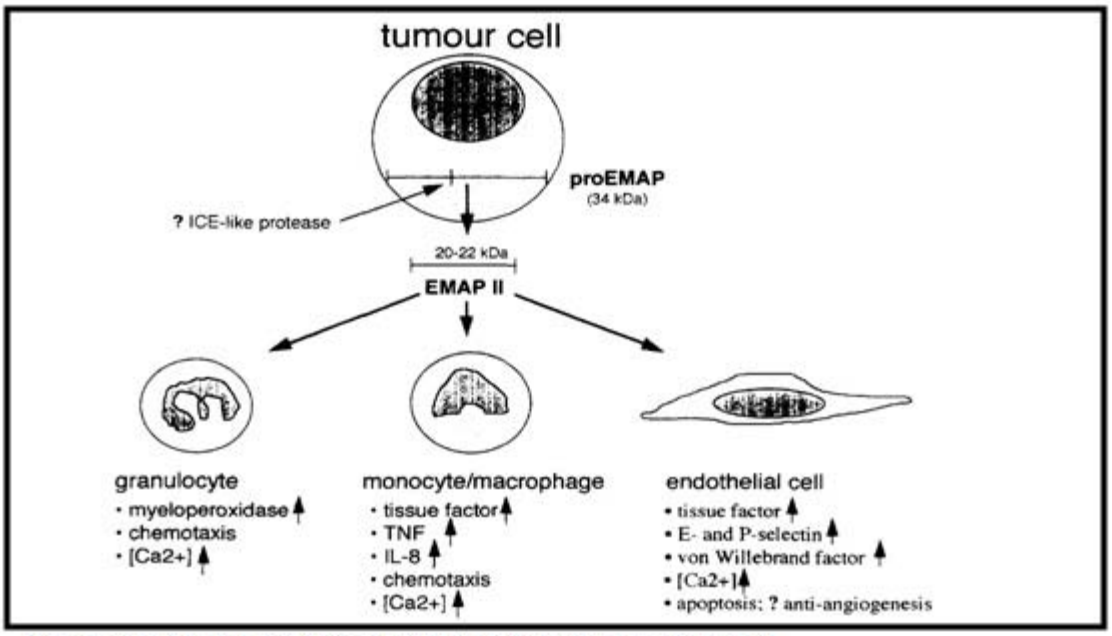


Figure 27: Model depicting EMAPII functions by Tas et al

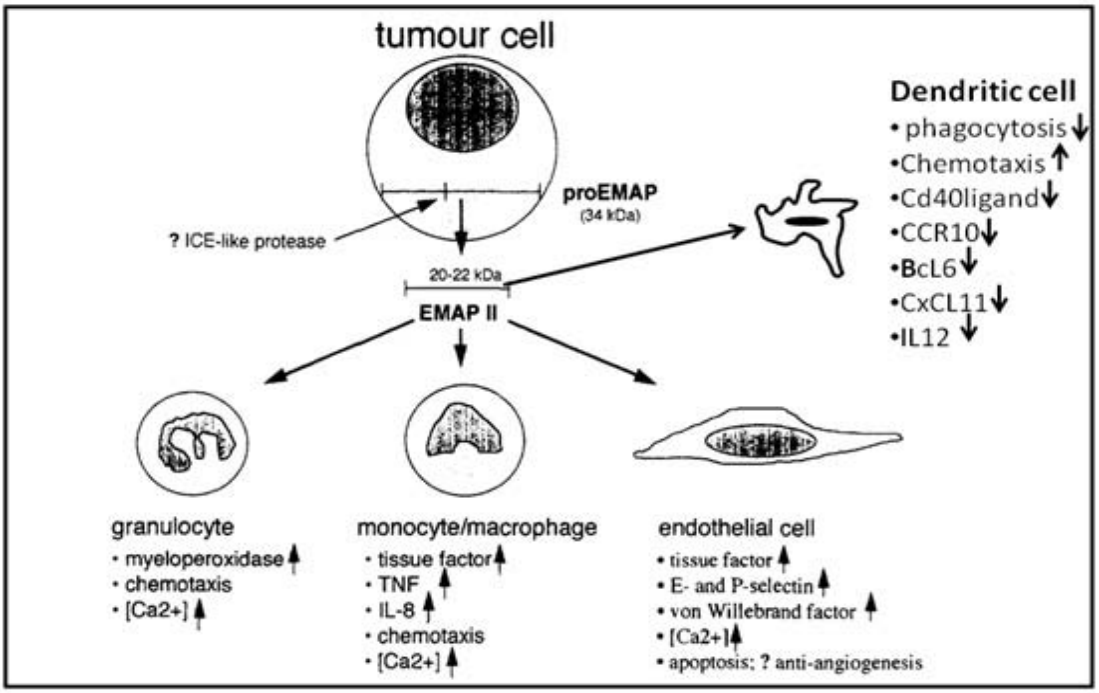


Figure 28: Modified model of the functions of EMAPII including the effects on DC
Model depicting EMAPII functions by Tas et al.

Considering the possible role played by EMAPII in peripheral tolerance, it can be used in treating autoimmunity. In conclusion, EMAPII is a multifunctional cytokine whose functions are exploited by tumors to benefit their survival. Our study does not support the use of this cytokine in treating tumor rather EMAPII has greater potential in being used as a therapy for auto immune diseases where in the signals required for mounting an auto immune response is hampered. Much work needs to be done to elucidate the potential of this cytokine in treating autoimmune disorders.

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APPENDICES

Appendix 1: Media Composition and Reagents

Complete JAWSII Growth Medium

Components	
RPMI 1640	
Fetal Bovine Serum/Bovine Growth Serum	5%
Penicillin	0.002 µg/ml
Streptomycin	0.2U/ml
Glutamine	2mM
Granulocyte-Macrophage Colony Stimulating factor(GM-CSF)	5-10 ng

SDS PAGE

5% Stacking Gel

For 5ml Solution	Volume in mls
29:1 Acrylamide/Bisacrylamide solution	0.83
1M Tris pH 6.8	0.62
20% Sodium Dodecyl Sulfate	0.025
Distilled water	2.9
Mix and degas for 10 minutes. Just before pouring the gel add	
20% Ammonium persulfate	0.025-0.05
TEMED	0.05-0.01

10% Resolving Gel

For 15ml Solution	Volume in mls
29:1 Acrylamide/Bisacrylamide solution	5
1M Tris pH 8.8	5.6
20% Sodium Dodecyl Sulfate	0.075
Distilled water	4.3
Mix and degas for 10 minutes. Just before pouring the gel add	
20% Ammonium persulfate	0.05
TEMED	10

5X Running Buffer

Glycine	72g
Trizma Base	15g
Sodium Dodecyl Sulfate	5g
Add distilled water to one liter	

5X Gel Loading Buffer

	Volume in mls
20% SDS	2.5
1M Tris pH 6.8	1.0
Distilled water	1.5

Glycerol	5
Bromophenol Blue Few grains	
Beta Mercaptoethanol (just before use)	50µl/ml

Western Blot

RIPA Lysis Buffer with protease and phosphatase inhibitors

NaCl	150mM
Triton X-100	1%
Sodium desoxycholate	0.5%
SDS	0.1%
Tris pH 8	50mM
Sodium meta vanadate Prepared stock solution of 100mM. Set the pH to 9.0, boiled and cooled repeatedly until pH stabilized at 9 and the solution turned colorless.	1mM
Sodium Azide	0.01%
PMSF (dissolved in isopropanol)	200µM
Aminocaproic acid	12.5mM
Benzaminidine	12.5mM

Transfer Buffer(pH 8.5)

Tris Base	*25mM*
Glycine	*0.2M*
Methanol	*20%*

Blocking Buffer

Tween 20	0.1%
Bovine Serum Albumin	5% w/v
1X Tris Buffered Saline	

Wash Buffer

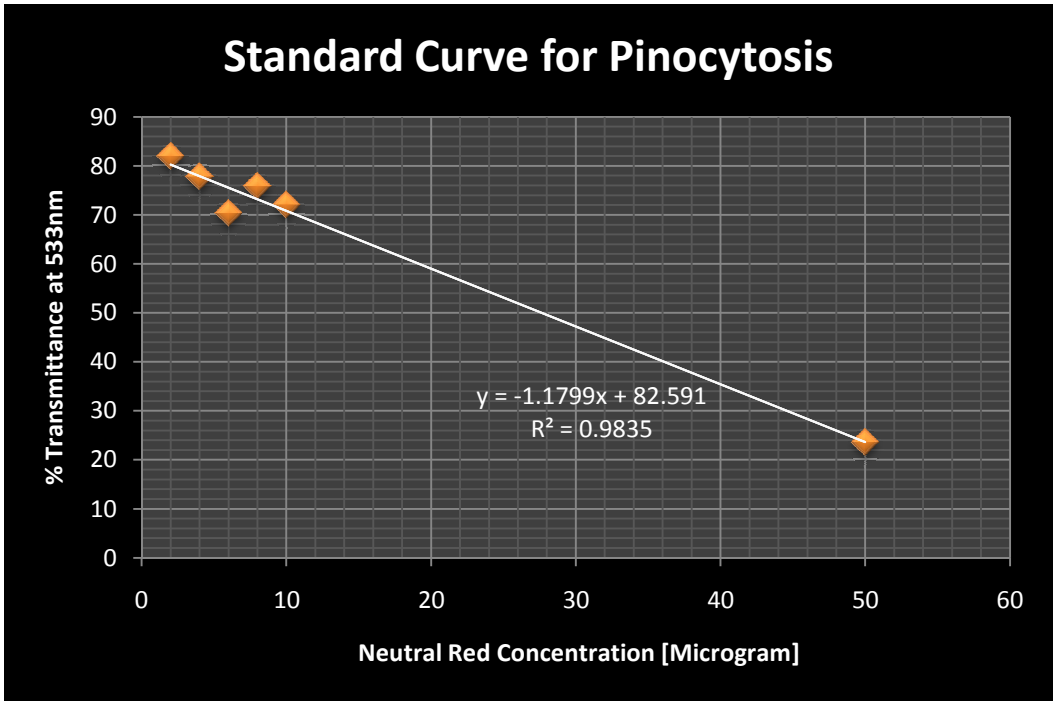
1X Tris Buffered Saline	
Tween 20	0.1%

Destaining Solution

Glacial Acetic Acid	10%
Methanol	10%

Appendix 2: Pinocytosis

1. Standard Curve Neutral Red versus Transmittance at 533nm.



2. Formula for µg neutral red/ 10^6 cells
   Concentration of neutral red/ml = µg neutral red obtained from the graph/5
   µg neutral red/ 10^6 cells = $\frac{10^6 \text{ x concentration of neutral red/ml}}{\text{total number of cells used}}$

VITA

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Scope and Method of Study:

EMAPII, a multi functional cytokine has been considered for anti-tumor therapy owing to its anti angiogenic properties. However, the effects of EMAPII on immune system cells like dendritic cells have not been studied. In this study, we analyzed the effects of EMAPII on dendritic cells that play a very important role in mounting anti tumor immunity. Various in vitro, in vivo and ex vivo studies were conducted to analyze the effect of EMAPII on dendritic cells.

Findings and Conclusions:

Endothelial Monocyte Activating Polypeptide (EMAPII) is chemotactic for dendritic cells. EMAPII reduces the release of T cell chemoattractants by dendritic cells and decreases the expression of cytokines such as IL12,IL3,IL7 that are important for activating T cells. It also down regulates the expression of CD40 ligand on dendritic cells that would render the associating T cells tolerogenic. Under normal conditions, EMAPII is released by apoptotic cells and plays an important role in inducing tolerance to self antigens. This property of EMAPII provides it with a potential to treat auto immune disorders. However, the ability of EMAPII to induce tolerance could be exploited by tumor cells. Induction of tolerance to self antigens would render tumor cells unsusceptible to immune clearance. From our data we conclude that EMAPII favors tumor survival and is not a suitable candidate for anti tumor therapy.

ADVISER'S APPROVAL: Dr. Kim Burnham